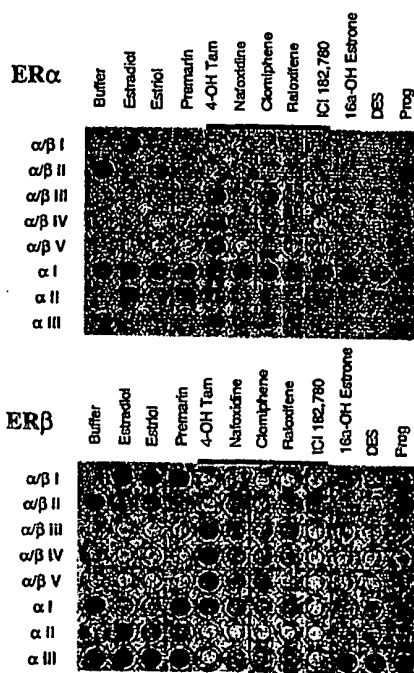


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**(57) Abstract**

The ability of a query compound to modulate the biological activity of a receptor in a multicellular organism is predicted on the basis of its interaction with that receptor in the presence of various member of a panel of BioKeys. The BioKeys are ligands, especially peptides or nucleic acids, known to modify the conformation of the receptor. This interaction data, known as a "fingerprint", is compared to the fingerprints for reference compounds with known biological activities mediated by that receptor. In the "molecular braille" (MB) embodiment of the present invention, the reference and test fingerprints are based on *in vitro* (cell-free) assays. In the "cellular-braille" (CB) embodiment of the present invention, the reference and test fingerprints are based on cellular assays (but not on assays of whole multicellular organisms, or their organs or tissues).

## Different Ligands Induce Different Structural Alterations in ER $\alpha$ and ER $\beta$



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METHOD OF PREDICTING THE ABILITY OF COMPOUNDS  
TO MODULATE THE BIOLOGICAL ACTIVITY OF RECEPTORS

Cross-Reference to Related Applications

Thorp, Serial No. 08/904,842, METHOD OF IDENTIFYING AND  
5 DEVELOPING DRUG LEADS WHICH MODULATE THE ACTIVITY OF A  
TARGET PROTEIN, discloses several methods of identifying  
drug leads. In essence a protein of interest, in one or  
more states, is characterized by (a) its chemical reactivity  
with one or more characterizing reagents, and/or (b) its  
10 binding to one or more aptamers (especially nucleic acids),  
generating an array of descriptors by which it may be  
characterized as more or less similar for reference proteins  
for which an equivalent array of descriptors have been  
generated, and for which one or more activity-mediating  
15 reference drugs are known. Suitable drug leads for the  
protein of interest are those analogous to the reference  
drugs for the more similar reference proteins.

Fowlkes, et al. PCT/US97/19638, 08/740,671, 09/050,359  
and 09/069,827, IDENTIFICATION OF DRUGS USING COMPLEMENTARY  
20 COMBINATORIAL LIBRARIES, disclose the use of a first  
combinatorial library, e.g., of peptides, to obtain a set of  
binding peptides that can serve as a surrogate for the  
natural ligand of a target protein. A small organic  
compound library (preferably combinatorial in nature) is  
25 then screened for compound which inhibit the binding of the  
surrogates to the target protein.

Paige, et al., Serial No. 60/082, 756, filed April 23,  
1998, and Paige, et al., Serial No. 60/099,656, filed  
September 9, 1998, are predecessors of the instant  
30 application.

All of the above applications are hereby incorporated  
by reference.

Mention of Government Support

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the invention.

## BACKGROUND OF THE INVENTION

### Field of the Invention

This invention relates to a method of identifying drugs  
5 which can mediate the biological activity of a target  
protein.

### Description of the Background Art

#### *Protein Binding and Biological Activity*

Many of the biological activities of the proteins are  
10 attributable to their ability to bind specifically to one or  
more binding partners (ligands), which may themselves be  
proteins, or other biomolecules.

When the binding partner of a protein is known, it is  
relatively straightforward to study how the interaction of  
15 the binding protein and its binding partner affects  
biological activity. Moreover, one may screen compounds for  
the ability of the compound to competitively inhibit the  
formation of the complex, or to dissociate an already formed  
complex. Such inhibitors are likely to affect the  
20 biological activity of the protein, at least if they can be  
delivered in vivo to the site of the interaction.

If the binding protein is a receptor, and the binding  
partner an effector of the biological activity, then the  
inhibitor will antagonize the biological activity. If the  
25 binding partner is one which, through binding, blocks a  
biological activity, then an inhibitor of that interaction  
will, in effect, be an agonist.

#### *Screening for Modulators of Receptor Activity*

The current state of the art for screening for  
30 modulators of receptor activity involves the displacement of  
a labeled ligand from the ligand binding pocket of the  
receptor. For example, a screen may be for displacement of  
radiolabeled estradiol from the estrogen receptor. This  
assay only provides information concerning the relative  
35 affinities of the compounds for the receptor and gives no



indication of the activity of the compound on the receptor, that is whether it functions as an agonist or an antagonist of receptor activity. This is a major problem for pharmaceutical companies to overcome in screening for  
5 modulators of receptor activity.

The assays that have been developed to date that can distinguish between agonists and antagonists involve cell-based assays and reporter gene systems. McDonnell, et al., Molec. Endocrinol., 9:659 (1995). In these systems, the  
10 receptor and a reporter gene are co-transfected into cells in culture. The reporter gene is only activated in the presence of active receptor. The ability of a compound to modulate receptor activity is determined by the relative strength of the reporter gene activity. These assays are  
15 time consuming and can produce variable results in different cell lines or with different reporter genes or response elements. Thus, the data must be interpreted with caution.

Methods have been developed that also take advantage of the different conformational states of receptors.  
20 Proteolytic digestion of the estrogen receptor in the presence of an agonist or antagonist produces distinct banding patterns on a denaturing polyacrylamide gel. In certain conformations, the receptor is protected from digestion at a particular site, while a different  
25 conformation may expose that site. Thus the banding patterns may indicate whether the receptor was complexed with an agonist or antagonist at the time of proteolytic digestion. This method requires copious amounts of receptor protein and is time consuming and expensive in that it  
30 requires a gel to be run for each sample. It is not suitable for screening numerous samples.

The following are examples of patents on cell based screening methods:

Patent #5723291 - Methods for screening compounds for  
35 estrogenic activity

Patent #5298429 - Bioassay for identifying ligands for steroid hormone receptors

Patent #5445941 - Method for screening anti-osteoporosis agents

Patent #5071773 - Hormone receptor-related bioassays

Patent #5217867 - Receptors: their identification,  
5 characterization, preparation and use

#### *Nuclear Receptors*

Nuclear receptors are a family of ligand activated transcriptional activators, see Evans and Hollenberg, Cell, 52:1-3 (1988), factors which include the receptors for  
10 steroid and thyroid hormones, retinoids, and vitamin D. The steroid receptor family is composed of receptors for glucocorticoids, mineralocorticoids, androgens, progestins, and estrogens. These receptors are organized into distinct domains for ligand binding, dimerization, transactivation,  
15 and DNA binding. Receptor activation occurs upon ligand binding, which induces conformational changes allowing receptor dimerization and binding of co-activating proteins. These co-activators, in turn, facilitate the binding of the receptors to DNA and subsequent transcriptional activation  
20 of target genes. In addition to the recruitment of co-activating proteins, the binding of ligand is also believed to place the receptor in a conformation that either displaces or prevents the binding of proteins that serve as co-repressors of receptor function. Lavinsky, et al., Proc.  
25 Nat. Acad. Sci. (USA), 95:2920 (1998).

The estrogen receptor is a member of the steroid family of nuclear receptors. Human ER $\alpha$  is a 595 amino acid protein composed of six functional domains or regions (A-F). The A/B region contains the transcription function AF-1, and the  
30 E domain contains the transcription function AF-2. These functions activate transcription in a cell- and promoter context-specific manner. AF-1 is constitutively active, while AF-2 is induced by hormone binding to the receptor. The C region contains the DNA-binding domain and a  
35 dimerization domain. The DNA-binding domain binds the estrogen (receptor) response element (ERE) associated with a regulated gene. The DBD contains two zinc fingers. The C

region may also be responsible for nuclear localization. The E region contains the hormone (ligand) binding domain.

The classical ERE is composed of two inverted hexanucleotide repeats, and ligand-bound ER binds to the ERE  
5 as a homodimer. The ER also mediates gene transcription from an AP1 enhancer element that requires ligand and the AP1 transcriptional factors Fos and Jun for transcriptional activation. Tamoxifen inhibits transcription of genes regulated by a classical ERE, but activates transcription of  
10 genes under the control of an AP1 element. See Paech, et al., Science, 277:1508-11 (1997).

In the absence of hormone, the estrogen receptor resides in the nucleus of target cells where it is associated with an inhibitory heat shock protein complex.  
15 (Smith, et al., (1993) Mol. Endocrinol., 7:4-11.) Upon binding ligand, the receptor is activated. This process permits the formation of stable receptor dimers and subsequent interaction with specific DNA response elements located within the regulatory region of target genes.  
20 (McDonnell, et al. (1991), Mol. Cell Biol., 11:4350-4355.) The DNA bound receptor can then either positively or negatively regulate target gene transcription. Although the precise mechanism by which the ER modulates RNA polymerase activity remains to be determined, it has been shown  
25 recently that agonist bound ER can recruit transcriptional adaptors, proteins that permit the receptor to transmit its regulatory information to the cellular transcriptional apparatus. (Onate, et al. (1995), Science, 270:1354-1357; Norris, et al. (1998), J. Biol. Chem., 273:6679-6688; Smith, et al. (1997), Mol. Endocrinol., 11:657-666). Conversely,  
30 when occupied by antagonists, the DNA bound receptor actively recruits co-repressors, proteins that permit the cell to distinguish between agonists and antagonists. (Norris, et al. (1998); Smith, et al. (1997); Lavinsky, et al., (1998) Proc. Natl. Acad. Sci. USA, 95:2920-2925).  
35 Building on this complexity was the recent discovery of a second estrogen receptor, ER $\beta$ , whose mechanism of action appears to be similar, yet distinct from ER $\alpha$ . (Greene, et

al. (1986), Science, 231:1150-1154; Kuiper, et al. (1996), Proc. Natl. Acad. Sci. USA, 93:5925-5930; Mosselman, et al. (1996), FEBS Lett., 392:49-53).

Thus, there are two forms of this receptor,  $\alpha$  and  $\beta$ ,  
5 presently known; other forms may exist. Both receptors activate transcription in response to estrogens, which are an important group of steroid hormones that not only influence the growth, differentiation, and functioning of the reproductive system, but also exert effects in the bone,  
10 brain and cardiovascular system. Estrogens can produce a broad range of effects in this diverse set of target tissues. These differential effects are believed mediated, in part, by tissue specific activation of the two different transactivation domains present at the amino-terminal and  
15 carboxy-terminal regions of the receptor. It is also likely that the two forms of the receptor ( $\alpha$  and  $\beta$ ) function in distinct tissues and thereby mediate the transactivation of different subsets of genes. (Paech, et al., Science, 277:1508, 1997; Kuiper and Gustafsson, FEBS Lett., 410:87,  
20 1997; Nichols, et al., EMBO J., 17:765, 1998; Montano, et al., Mol. Endo., 9:814, 1995.)

Drugs that target the estrogen receptor can exhibit a variety of effects in different target tissues. For example, tamoxifen is an ER antagonist in breast tissue,  
25 (Jordan, V.C., (1992) Cancer, 70:977-982), but an ER agonist in bone (Love, et al. (1992), New Engl. J. Med., 326:852-856) and uterine, (Kedar, et al. (1994), Lancet, 343:1318-1321) tissue. Raloxifene is also an ER antagonist in breast tissue; however, it exerts agonist activity in bone but not  
30 uterine tissue (Black, et al. (1994), J. Clin. Invest., 93:63-69). Indeed, one of the greatest challenges in understanding the pharmacology of the estrogen receptor is determining how different ER ligands produce such diverse biological effects.

35 Estrogens, in general, are stimulatory agents, resulting in increased gene expression and cell proliferation in target tissues. However, many molecules have been described that bind to the estradiol binding site

on the receptor, but produce negative effects on gene expression and cell growth. These agents have historically been termed "antiestrogens", but this term has proven to be much too simplistic. (Tremblay, et al., Can. Res., 58:877, 1988; Katzenellenbogen, et al., Breast Can Res. Treatm., 44:23, 1997; Howell, Oncology (suppl. 1), 11:59, 1997; Gallo and Kaufman, Sem. in Oncol. (Suppl. 1), 24:71, 1997). One of the most noteworthy of these agents is tamoxifen, which has been successfully used in the treatment of ER-positive breast cancer. Tamoxifen, a derivative of triphenylethylene, is metabolized in the cell to produce 4-OH tamoxifen, which has very high affinity for the estradiol binding pocket of the ER. Although this compound competes with estradiol for binding to the ER, it does not induce transcriptional activation in breast tissue, thus it does not promote cell growth and acts as a classic antiestrogen in this tissue. Tamoxifen, however, does have estrogen-like activities in other tissues. In the uterus, tamoxifen acts as an agonist of receptor activity, stimulating the growth of uterine tissue leading to an increased incidence of endometrial hyperplasia in treated patients. Tamoxifen also produces estrogenic effects in the bone and cardiovascular system. This activity generates beneficial effects such as reducing the risk of osteoporosis and lowering serum LDL levels. The numerous differential effects produced by compounds such as tamoxifen has led to the replacement of the term "antiestrogen" with "selective estrogen receptor modulators" or SERMs. SERMs may have both positive and negative effects on ER activity depending on the biology of receptor and the tissue in which it is being expressed.

A goal of current research is to develop SERMs that have agonistic or estrogenic effects on bone and the cardiovascular system and antagonistic or antiestrogenic effects in the breast and uterus. One SERM that has recently been approved for treatment of post-menopausal symptoms is Raloxifene. Raloxifene is a benzothiophene derivative that, like tamoxifen, binds in the ligand binding pocket of the ER. Clinical studies indicate that this

compound lacks estrogenic activity in the breast and uterus, but produces estrogenic activity in the bone and perhaps the cardiovascular system. It is currently prescribed for prevention for osteoporosis in post-menopausal women. There  
5 are several additional SERMs in clinical trials, and a great deal of effort in the pharmaceutical industry is focused on the identification and characterization of additional SERMs.

The search for SERMs poses a major obstacle. In order to screen large libraries of compounds for SERMs, it is  
10 necessary to have a convenient assay for identifying which lead molecules have the desired effect(s). Currently, when a compound is identified that competes with estradiol for binding to the ER, a number of cell-based assays must be conducted to determine its activity. These studies are more  
15 laborious than in vitro assays and still do not absolutely predict the complete spectrum of biological activity of the SERM. Thus, studies often have to move into animal models or clinical trials before the selective modes of action of the SERM can be determined. A simple *in vitro* system to  
20 distinguish between agonist and antagonist activity of a SERM would be of great utility.

The development of such a system requires knowledge of the mechanisms that produce the broad effects of SERMs. There is evidence that SERMs are able to produce  
25 differential (agonistic and antagonistic) effects due to their ability to alter the conformation of the ER. In general, the receptor is thought of as having two conformations, active or inactive. These conformations are formed in the presence or absence of ligand, respectively.  
30 The SERM drives the receptor into a conformation that is neither fully active nor fully inactive. This intermediate conformation creates changes in the association patterns of co-activators, co-repressors, and other regulatory molecules with the receptor, thus producing variable effects. The  
35 broad range of effects produced by SERMs may also be due to selective tissue expression of ER alpha and beta as well as co-activators and co-repressors. It may also be due to different affinities of the SERM for the two receptors.

### *Traditional Drug Screening*

In traditional drug screening, natural products (especially those used in folk remedies) were tested for biological activity. The active ingredients of these products were purified and characterized, and then synthetic analogues of these "drug leads" were designed, prepared and tested for activity. The best of these analogues became the next generation of "drug leads", and new analogs were made and evaluated.

Both natural products and synthetic compounds could be tested for just a single activity, or tested exhaustively for any biological activity of the interest to the tester. Testing was originally carried out in animals, later, less expensive and more convenient model systems, employing isolated organs, tissue, or cells, or cell cultures, membrane extracts or purified receptors, were developed for some pharmacological evaluations.

Testing in whole animals and isolated organs typically requires large amounts of chemical compound to test. Since the quantity of a given compound within a collection of potential medicinal compounds is limited, this requires one to limit the number of screens executed.

Also, it is inherently difficult to establish structure/activity relationships (SAR) among compounds tested using whole animals, or isolated organs or tissues or, to a lesser extent, cultured cells. This is because the actual molecular target of any given compound's action may be quite different from that of other compounds scoring positive in the assay. By testing a battery of compounds on a very specific target, one can correlate the action of various chemical residues with the quantitative activity and use that information to focus ones search for active compounds among certain classes of compounds or even direct the synthesis of novel compounds having a composite of the properties shared by the active compounds tested.

Another disadvantage to whole animal, organ, tissue and cell based screening is that certain limitations may prevent an active compound from being scored as such. For instance,

an inability to pass through the cellular membrane may prevent a potent inhibitor, within a tested compound library, from acting on the activated oncogene ras and giving a spurious negative score in a cell proliferation assay. However, if it were possible to test ras in an isolated system, that potent inhibitor would be scored as a positive compound and contribute to the establishment of a relevant SAR. Subsequent, chemical modifications could then be carried out to optimize the compound structure for membrane permeability. (In the case of cell-based assays, this problem can be alleviated to some degree by altering membrane permeability.)

*Drug Discovery.* The human genomics effort could yield gene sequences that code for as many as 70,000 proteins, each a potential drug target; microbial genomics will increase this number further. Unfortunately, since genomic studies identify genes, but not the biological activity of the corresponding proteins, it is likely that many of the genes will prove to encode proteins whose activation or inactivation has no effect on disease progression. (Gold, et al., J. Nature Biotech., 15:297, 1997). There is therefore a need for a method of determining which proteins are most likely to be productive targets for pharmacological intervention.

Even if one knew in advance the perhaps 10,000 proteins which could be considered interesting targets, there remains the problem of efficiently screening hundreds of thousands of possible drugs for a useful activity against these 10,000 targets.

Historically, acquiring chemical compound libraries has been a barrier to the entry of smaller firms into the drug discovery arena. Due to the large quantity of chemical required for testing on whole animals and even on cells in culture, it was a given that whenever a compound was synthesized it should be done in fairly large quantity. Thus, there was a synthesis and purification throughput of less than 50 compounds per chemist per year. Large companies maintained their immensely valuable collections as



trade barriers. However, with the downsizing of targets to the molecular level and the automation of screens, the quantity of a given compound necessary for an assay has been reduced to very small amounts. These changes have opened the door for the utilization of so-called combinatorial chemistry libraries in lieu of the traditional chemical libraries. Combinatorial chemistry permits the rapid and relatively inexpensive synthesis of large numbers of compounds in the small quantities suitable for automated assays directed at molecular targets. Numerous small companies and academic laboratories have successfully engineered combinatorial chemical libraries with a significant range of diversity (reviewed in Doyle, 1995, Gordon et al, 1994a, Gordon et al, 1994b).

15        *Combinatorial Libraries.* In a combinatorial library, chemical building blocks are randomly combined into a large number (as high as  $10^{15}$ ) of different compounds, which are then simultaneously screened for binding (or other) activity against one or more targets.

20        Libraries of thousands, even millions, of random oligopeptides have been prepared by chemical synthesis (Houghten et al., Nature, 354:84-6(1991)), or gene expression (Marks et al., J Mol Biol, 222:581-97(1991)), displayed on chromatographic supports (Lam et al., Nature, 25    354:82-4(1991)), inside bacterial cells (Colas et al., Nature, 380:548-550(1996)), on bacterial pili (Lu, Bio/Technology, 13:366-372(1990)), or phage (Smith, Science, 228:1315-7(1985)), and screened for binding to a variety of targets including antibodies (Valadon et al., J Mol Biol, 30    261:11-22(1996)), cellular proteins (Schmitz et al., J Mol Biol, 260:664-677(1996)), viral proteins (Hong and Boulanger, Embo J, 14:4714-4727(1995)), bacterial proteins (Jacobsson and Frykberg, Biotechniques, 18:878-885(1995)), nucleic acids (Cheng et al., Gene, 171:1-8(1996)), and 35    plastic (Siani et al., J Chem Inf Comput Sci, 34:588-593(1994)).

Libraries of proteins (Ladner, USP 4,664,989), peptoids

(Simon et al., Proc Natl Acad Sci U S A, 89:9367-71(1992)), nucleic acids (Ellington and Szostak, Nature, 246:818(1990)), carbohydrates, and small organic molecules (Eichler et al., Med Res Rev, 15:481-96(1995)) have also  
5 been prepared or suggested for drug screening purposes.

The first combinatorial libraries were composed of peptides or proteins, in which all or selected amino acid positions were randomized. Peptides and proteins can exhibit high and specific binding activity, and can act as  
10 catalysts. In consequence, they are of great importance in biological systems. Unfortunately, peptides per se have limited utility for use as therapeutic entities. They are costly to synthesize, unstable in the presence of proteases and in general do not transit cellular membranes. Other  
15 classes of compounds have better properties for drug candidates.

Nucleic acids have also been used in combinatorial libraries. Their great advantage is the ease with which a nucleic acid with appropriate binding activity can be  
20 amplified. As a result, combinatorial libraries composed of nucleic acids can be of low redundancy and hence, of high diversity. However, the resulting oligonucleotides are not suitable as drugs for several reasons. First, the oligonucleotides have high molecular weights and cannot be  
25 synthesized conveniently in large quantities. Second, because oligonucleotides are polyanions, they do not cross cell membranes. Finally, deoxy- and ribo-nucleotides are hydrolytically digested by nucleases that occur in all living systems and are therefore usually decomposed before  
30 reaching the target.

There has therefore been much interest in combinatorial libraries based on small molecules, which are more suited to pharmaceutical use, especially those which, like benzodiazepines, belong to a chemical class which has  
35 already yielded useful pharmacological agents. The techniques of combinatorial chemistry have been recognized as the most efficient means for finding small molecules that act on these targets. At present, small molecule

combinatorial chemistry involves the synthesis of either pooled or discrete molecules that present varying arrays of functionality on a common scaffold. These compounds are grouped in libraries that are then screened against the target of interest either for binding or for inhibition of biological activity. Libraries containing hundreds of thousands of compounds are now being routinely synthesized; however, screening these large libraries for binding or inhibition with all 10,000 potential targets cannot be reasonably accomplished with present screening technologies, and there are numerous experimental and computational strategies under development to reduce the number of compounds that must be screened for each target.

*Information-intensive drug discovery.* As pointed out by Paterson, et al., J. Med. Chem., 39: 3049-59 (1996), medicinal chemistry advances through the dual processes of "lead discovery" and "lead optimization". In "lead discovery", the search objective is the discovery of an "activity island", a chemical class with a high frequency of active molecules. (this class may be defined mathematically as a volume within a multidimensional space defined by various molecular descriptors). In "lead optimization", the "activity island" is explored in detail. If each compound synthesized and tested can be considered as a probe of a "neighborhood" of similar compounds, in "lead discovery", it is inefficient to test substances whose neighborhoods overlap.

Coupled to the recent advancements in genomics and molecular biology has been a revolution in information technology, which includes relational databases, computer graphics, and neural networks (13). These capabilities permit the construction of databases of descriptors that describe either compounds or targets in quantitative terms, and these descriptors can be related to make predictions about the structures of compounds, their biological activities, and the targets they act on (5-8).

Structure descriptors can be based on a variety of structural features. These approaches provide arrays of

molecular descriptors that can be used to assess the similarity of molecules in a library.

See Patterson, et al. , et al., J. Med. Chem., 39: 3049-59 (1996), Klebe and Abraham, J. Med. Chem., 36:70-80 (1993), Cummins, et al., J. Chem. Inf. Comput. Sci., 36:750-63 (1996), Matter, J. Med. Chem., 40:1219-29 (1997); Weinstein, et al., Science, 275:343-9 (1997).

For proteins, structural descriptors cannot be directly calculated from the amino acid sequence.

10       Compounds may be characterized by their activity rather than by structure. Kauvar, et al., Chemistry & Biology, 2: 107-118 (1995) "fingerprinted" over 5,000 compounds by the binding potency (concentration needed to inhibit 50% of the protein's activity) of each compound to each member of a  
15       reference panel of eight proteins. (These proteins were selected on the basis of readily assayable activity, broad cross-reactivity with small organic molecules, and low correlation between each other in binding patterns.) A screening library of 54 compounds was then selected based on  
20       the diversity in their "fingerprints" (inhibitory activity against the reference panel proteins).

      This "training set" was used to evaluate the similarity of the ligand binding characteristics of a new protein to one of the reference panel proteins. By regression  
25       analysis, a computational surrogate (a weighted sum of two or more reference panel proteins) for the new protein is determined. The activity of all fingerprinted compounds to inhibit the activity of the new protein is predicted as the sum of their appropriately weighted inhibitory activities  
30       against the component reference proteins of the computational surrogate. Predictions may be improved by testing additional sets of compounds against the new protein. See also L. M. Kauvar, H. O. Villar. Method to identify binding partners. US Patent 5587293.

35       Weinstein, supra, in a study of the molecular pharmacology of cancer, took a similar approach. The "activity" database (A) contains the activities against 60 cell lines for 60,000 compounds that have been screened at

NCI. The similarity in the activity profile against the panel of cell lines can then be calculated for any two compounds, and is generally assessed by a pairwise correlation coefficient (PCC), which is determined by an algorithm called COMPARE, which calculates the similarity of all of the compounds in the database to a user-supplied "seed" compound.

All references, including any patents or patent applications, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert and applicants reserve the right to challenge the accuracy and pertinency of the cited document.

**SUMMARY OF THE INVENTION**

The present invention is directed to a method for the more efficient identification of small organic molecules, preferably molecules having a molecular weight of less than 500 daltons, which are pharmaceutically acceptable and which are potent modulators of the biological activity of a protein.

This method provides a simple and consistent means for identifying and characterizing modulators of receptor activity, using oligomers (especially peptides) to probe receptor conformation. It can be used as both a tool in both primary and secondary screens for compounds that modulate the activity of a receptor. In some embodiments, the method is also completely *in vitro* so the activity of a compound can be assessed without using a cell based assay, let alone a whole animal assay.

We have explored the possibility that various ER ligands induce distinct conformational changes in the ER. These distinct conformations may, in turn, alter the interactions of the receptor with cell and tissue specific co-activating or co-repressing proteins or even estrogen response elements, thus leading to diverse biological effects. Using limited proteolysis, we and others have shown that the ER agonist estradiol and the ER antagonist Imperial Chemical Industries (ICI) 162,780 induced distinct ER conformations (McDonnell, et al. (1995), Mol. Endocrinol., 9:659-669; Beekman, et al. (1993), Mol. Endocrin., 7:1266-1274). However, the picture is much more complicated than this. There are a variety of ER ligands, namely, selective estrogen receptor modulators (SERMs), which are neither pure agonists nor antagonists. These ligands, which include tamoxifen and raloxifene, produce distinct tissue specific biological effects, yet conformational differences cannot be discerned in the protease digestion assay. It is likely that these compounds are also eliciting distinct conformational changes that affect ER activity, but the changes are too subtle to be detected by the protease digestion assay (Brzozowski, et al.

(1997), Nature, 389:753-758; Shiau, et al. (1998), Cell, 95:927-937).

This invention is based on the observation that peptides isolated by screening a phage-displayed peptide library for binding to the estrogen receptor had dramatically different binding affinities depending on whether the receptor was unliganded, or complexed with an agonist or an antagonist. Thus, the peptide binding appears to be a barometer of protein conformation, and hence of whether a compound which is complexed to the receptor is acting as an agonist or an antagonist.

In essence, a panel of "BioKeys" (typically peptides) which alter the conformation of a receptor in distinctly different ways, are used to obtain a "fingerprint" of how a compound of interest interact with that receptor in its various BioKey-modified conformations, each element of the fingerprint being a measure of the strength of interaction of the compound with the receptor in the presence of a given BioKey. Once fingerprints are obtained for a reasonable number of reference compounds with known biological activities, as measured by a "gold standard" (whole animal, or isolated organ or tissue) assay, the similarity of the fingerprint of a new compound to that of the reference compounds may be calculated, and used to predict the bioactivity of the new compound.

The invention has advantages over the whole animal-based systems described above in that 1) the same technology can be applied to a variety of different receptors, 2) the system can be used for high throughput screening and compound characterization, and 3) the system gives very distinct patterns for agonists and antagonists of receptor activity using very little protein.

In the "molecular braille" (MB) embodiment of the present invention, the reference and test fingerprints are based on in vitro (cell-free) assays.

In the "cellular-braille" (CB) embodiment of the present invention, the reference and test fingerprints are based on cellular assays (but not on assays of whole

multicellular organisms, or their organs or tissues).

The advantages of "molecular braille" are

- gives information about affinity, and, based on a fingerprint, bioactivity in a single assay
- 5 ● can be faster and less expensive if the protein is a) inexpensive to purchase or b) easy to express and purify
- gives information about structure-activity relationships
- 10 ● peptide/receptor interactions may be more sensitive because there will not be anything extraneous to get in the way

Its disadvantages are

- protein may not be properly folded, modified, or be in the presence of cofactors it needs to be active
- 15 ● doesn't give much of the information given by CB

In contrast, the advantages of "cellular braille" are

- If in yeast it can be cheaper than MB
- 20 ● Bioactivity (including dose:effect) information
- gives closer indication of how a whole animal might respond
- you may get active metabolites
- no need for protein purification

25 Its disadvantages are

- compounds that cannot get into the cell will automatically be selected against
- does not give affinity information directly
- throughput likely to be lower than with MB,
- 30 although still better than whole animal assay.

Both "molecular braille" and "cellular braille" are faster and less expensive than whole animal bioassays, and more readily automated for high throughput, and their use as preliminary screens helps minimize experimentation on  
35 animals, which itself is an ethical goal of society.



It will be appreciated that both techniques may be used, either sequentially or simultaneously. For example, MB may be used as a first screen and CB as a second screen of the first round positives. Or compounds may be screened  
5 by both MB and CB, and compounds earmarked by either screen given further attention. Similarities may be calculated separately from in vitro and cell-based assays, or the results of these two types of assays may be combined into a single fingerprint for each reference or test compound.

10 In a preferred embodiment, this method uses phage display to isolate peptides (BioKeys) that map the sites of biological interaction on both the active and the inactive receptor. These BioKeys are probes for alterations in receptor conformation, and can readily distinguish between  
15 active, inactive and partially active receptor. The patterns of binding obtained with the peptides provides a fingerprint of the receptor conformation. The binding of the individual peptides will increase or decrease in the presence of an agonist or an antagonist of receptor  
20 activity. Such activity may or may not be tissue-specific. In some cases, whether a molecule is an agonist or an antagonist will depend on the tissue in question (e.g. for SERMs), or on other environmental factors. Therefore, the peptides may be used to classify compounds, not only as pure  
25 agonists or antagonists, but also more complexly. The method has the following applications:

1) One or more of these peptides can be used in a competitive displacement assay to identify modulators of receptor activity in a high-throughput (in vitro or simple  
30 cell) screen.

2) The peptides can be used to fingerprint modulators of receptor activity and classify them as agonists or antagonists of receptor activity.

3) Peptides identified for orphan receptors may  
35 be used to identify the natural ligand of these receptors.

4) This method may be used for nuclear receptors as well as other receptors such as G-protein coupled receptors.

5) Method can be applied to any protein that undergoes a conformational change upon ligand/substrate binding.

In a particular preferred embodiment, the invention is used to predict SERM activity against nuclear receptors, such as the estrogen receptor.

In order to characterize SERM activity at the estrogen receptor, we have developed a system that utilizes peptides to mimic the binding of various ER associated proteins to ER  $\alpha$  and  $\beta$  in an *in vitro* setting. The peptides bind preferentially to either the active or inactive conformation of the receptor, and will distinguish between different conformational changes in the ER that result from the binding of a SERM. The system will also allow the comparison of effects of the SERM on ER  $\alpha$  and  $\beta$ . This assay provides a simple procedure to determine the relative agonist/antagonist activity of a newly identified SERM. The technology may also be applied to the analysis of selective modulators of any receptor.

We have developed an *in vitro* system for identifying, characterizing and classifying modulators of receptor activity. The technique was developed using the estrogen receptor and is based on mapping sites of biological interaction on the active and inactive receptor using phage displayed peptide libraries. The peptides that bind to these sites appear to mimic proteins that bind preferentially to the active or inactive estrogen receptor. Certain sites on the receptor are only available for binding when an agonist is bound to the ER. Other sites are more readily available for binding with a SERM complexed ER. The relative binding affinities of these peptides on an estrogen complexed receptor, or a SERM complexed receptor relative to an unliganded receptor provides a fingerprint that is indicative of the agonist/antagonist activity of the SERM. The system has been tested on the ER using several known agonists and SERMs. Agonists of receptor function and SERMs produced distinct fingerprints in our system indicative of their distinct *in vivo* functions. This system may be used

as a primary screening tool to identify hits, to classify lead compounds from a drug screen, to characterize SERMs in terms of agonist and antagonist function and to predict possible clinical effects of SERMs such as tissue and  
5 receptor specificity. This method can also be applied to the fractionation of mixtures of SERMs to determine which components are producing agonistic and antagonistic activity. This method may also be used with other receptors (e.g., progesterone, androgen, glucocorticoid, thyroid,  
10 vitamin D, beta-adrenergic, dopamine, epidermal growth factor, etc.), to identify, characterize and classify modulators of receptor activity.

While peptides have been identified for use as probes to modify receptor conformation, to help screen compound  
15 libraries, certain of these peptides may be useful in their own right as drugs or diagnostics.

In addition, nonpeptide mimetics or other analogues of the aforementioned peptides may be useful as drugs or  
diagnostics.

20 The screened compounds, and their analogues, are also of interest.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effect of seven drugs which modulate estrogen receptor activity, in five sites of action.

Figure 2 maps the binding site for interaction of the  
5 four peptides with ER $\alpha$  (or moieties thereof) as influenced by estradiol or 4-OH tamoxifen.

Figure 3 shows that different ligands induce different structural alterations in ER alpha and ER beta, as shown by differences in the binding of 11 different peptides. (The  
10 data in this figure is tabulated in table 14.)

Figure 4 compares the effects of estradiol (an agonist) and raloxifene (an antagonist) on ERalpha conformation.

Figure 5 shows how the data in Fig. 3 and Table 14A is used to calculate similarities.

15 Figure 5A duplicates Table 14A.

Figure 5B shows the raw Euclidean distances.

Figure 5C shows the calculated similarities, after scaling:

$$20 \quad \text{similarity} = \frac{\text{maximum dist} - \text{actual dist}}{\text{maximum dist}}$$

With 8 descriptors (BioKeys) and scores 0-7, the maximum distance is SQRT (7\*7\*8), or 19.79899.

Figure 5D is a 3D bar graph corresponding to 5C.

25 Figure 5E is a 2D bar graph isolating the similarity data for estradiol and 4-OH tamoxifen.

Figure 6 shows, in a similar manner, the calculation of similarities based on the ERbeta data.

Figure 7 analyzes the interaction of seven drugs with  
30 ER $\alpha$  and four different peptides (AB1, A2, AB3, AB5) using a mammalian two-hybrid assay system.

Figure 8 analyzes the specificity of interaction of various drugs with four more nuclear receptors and the same four peptides using the same assay system.

35 Figure 9 explores the interaction of the four peptides with mutant receptors (impaired AF-2 function) as influenced by seven different drugs.

Figure 10 studies the disruption of ER mediated transcriptional activity as a function of peptide

concentration.

Figure 11 shows that the A2 disruption of tamoxifen-activated ER is not promoter-dependent.

Figure 12 explores disruption of ER transcriptional activity as mediated by the AP-1 pathway.

Figure 13 is a schematic model of potential mechanisms of action of peptides which block tamoxifen partial agonist activity.

Figure 14 shows the normalized luciferase activity of a two-hybrid mammalian system for ER AF2 in presence of estradiol (E2), 4-OH tamoxifen, ICI, DES, GW 7604, estrone, equilin and D8,9DHE.

Figure 15 shows the binding of various peptides to both wild-type and mutant ER.

Figure 16 A shows the disruption of E $\alpha$  transcriptional activation function in mammalian cells as a result of the action of LXXLL-containing peptides. B. shows the synergistic interaction of two copies of LXXLL motif function to compete with endogenous coactivators.

Figure 17 shows that LXXLL containing peptides disrupt AF2 functions in HepG2 cells.

Figure 18 shows that nuclear receptors have distinct preferences for different peptides with LXXLL motifs.

Figure 19 shows that peptide 293 selectively disrupts Erb dependent reporter gene expression without affecting Era dependent transcription.

Figure 20 shows a similarity analysis of the data pictured in Figure 7.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS  
OF THE INVENTION

Receptor-Mediated Pharmacological Activity

Many pharmacologically active substances elicit a  
5 specific physiological response by interacting with an  
element, known as a receptor, of the target cell. A  
receptor is a component, usually macromolecular, of an  
organism with which a chemical agent interacts in some  
specific fashion to cause an action which leads to an  
10 observable biological effect. For purposes of the present  
invention, antibodies are not considered receptors.

The substances which are able to elicit the  
response, by specific interaction with a receptor site, are  
known as agonists. Typically, increasing the concentration  
15 of the agonist at the receptor site leads to an increasingly  
larger response, until a maximum response is achieved. A  
substance able to elicit the maximum response is known as a  
full agonist, and one which elicits only, at most, a lesser  
(but discernible) response is a partial agonist.

20 A pharmacological antagonist is a compound which  
interacts with the receptor without eliciting a response,  
and by doing so inhibits the receptor from responding to  
agonists. A competitive antagonist is one whose effect can  
be overcome by increasing the agonist concentration; a  
25 noncompetitive antagonist is one whose action is unaffected  
by agonist concentration. A sequestering antagonist is one  
which inhibits a ligand: receptor interaction by binding to  
the ligand in such a way that it can no longer bind the  
receptor. A competitive sequestering antagonist competes  
30 with the receptor for the ligand, whereas a competitive  
pharmacological antagonist competes with the ligand for the  
receptor.

Ligands are substances which bind to receptors,  
and thereby encompass both agonists and pharmacological  
35 antagonists. However, ligands exist which bind receptors,  
but which neither agonize nor antagonize the receptor.  
Ligands which activate (agonize) or inhibit (antagonize) the

receptor are here collectively termed modulators. Some modulators change roles, acting as agonists or antagonists, depending on circumstances.

Natural ligands are those which, in nature,  
5 without human intervention, are responsible for agonizing or antagonizing a natural receptor. A natural ligand may be produced by the organism to which the receptor is native. A ligand native to a pathogen or parasite may bind to a receptor native to a host. Or a ligand native to a host may  
10 bind to a receptor native to a pathogen or parasite. All of these are natural ligands.

The clinical concept of drug antagonism is broader than the pharmacological concept, including phenomena that do not involve direct inhibition of agonist:receptor  
15 binding. A "physiological" antagonist could be a substance which directly or indirectly inhibits the production, release or transport to the receptor site of the natural agonist, or directly or indirectly facilitates its elimination (whether physical, or by modification to an  
20 inactive form) from the receptor site, or inhibits the production or increases the rate of turnover of the receptor, or interferes with signal transduction from the activated receptor.

A physiological antagonist of one receptor (e.g.,  
25 an estrogen receptor) may be a pharmacological antagonist of another, e.g., a transcription factor. A physiological antagonist of one receptor may be a pharmacological agonist of another receptor, such as one which activates an enzyme which degrades the natural ligand of the first receptor.

30 Similarly, one may speak of a physiological agonist, which is a substance which directly or indirectly enhances the production, release or transport to the receptor site of the natural agonist, or directly or indirectly inhibits its elimination from the receptor site,  
35 or enhances the production or reduces the rate of turnover of the receptor, or in some way facilitates signal transduction from the activated receptor.

It follows that there are both "pharmacological"

and "physiological" modulators.

A functional antagonist of a receptor is a substance which acts on a second receptor triggering a biological response which counteracts or inhibits the normal response to activation of the first receptor. Thus, a functional antagonist of one receptor may be a pharmacological agonist of another.

If a disease state is the result of inappropriate activation of a receptor, the disease may be prevented or treated by means of a physiological or pharmacological antagonist. Other disease states may arise through inadequate activation of a receptor, in which case the disease may be prevented by means of a suitable physiological or pharmacological agonist.

An important class of receptors are proteins embedded in the phospholipid bilayer of cell membranes. The binding of an agonist to the receptor (typically at an extracellular binding site) can cause an allosteric change at an intracellular site, altering the receptor's interaction with other biomolecules. The physiological response is initiated by the interaction with this "second messenger" (the agonist is the "first messenger") or "effector" molecule.

Enzymes are special types of receptors. Receptors interact with agonists to form complexes which elicit a biological response. Ordinary receptors then release the agonist intact. With enzymes, the agonists are enzyme substrates, and the enzymes catalyze a chemical modification of the substrate. Thus, enzyme substrates are "ligands". Enzymes are not necessarily integral membrane proteins; they may be secreted, or intracellular, proteins. Often, enzymes are activated by the action of a receptor's second messenger, or, more indirectly, by the product of an "upstream" enzymatic reaction.

Thus, drugs may also be useful because of their interaction with enzymes. The drug may serve as a substrate for the enzyme, as a coenzyme, or as an enzyme inhibitor. (An irreversible inhibitor is an "inactivator".) Drugs may



also cause, directly or indirectly, the conversion of a proenzyme into an enzymes. Many disease states are associated with inappropriately low or high activity of particular enzymes.

5           The present invention may be used to identify both agonists and antagonists of receptors. It is not unusual for a relatively small structural change to convert an agonist into a pharmacological antagonist, or vice versa. Therefore, even if the drugs known to interact with a  
10 reference protein are all agonists, the drugs in question may serve as leads to the identification of both agonists and antagonists of the reference protein and of related proteins. Similarly, known antagonists may serve as drug leads, not only to additional antagonists, but to agonists  
15 as well.

#### Potency

The potency of an antagonist of a receptor may be expressed as an IC<sub>50</sub>, the concentration of the antagonist which causes a 50% inhibition of a receptor's binding or  
20 biological activity in an in vitro or in vivo assay system. A pharmaceutically effective dosage of an antagonist depends on both the IC<sub>50</sub> of the antagonist, and the effective concentrations of the receptor and its clinically significant binding partner(s).

25           Potencies may be categorized as follows:

	<u>Category</u>	<u>IC<sub>50</sub></u>
	Very Weak	>1 $\mu$ moles
	Weak	100 n moles to 1 $\mu$ mole
	Moderate	10 n moles to 100 n moles
30	Strong	1 p mole to 10 n moles
	Very Strong	<1 p mole

Preferably, the antagonists identified by the present invention are in one of the four higher categories identified above, and are in any event more potent than any  
35 antagonist known for the protein in question at the time of filing of this application.

In a similar manner, the potency of an agonist may

be quantified as the dosage resulting in 50% of its maximal effect on a receptor.

#### General Method

In the present invention, the biological activity  
5 of a test substance, as mediated by a particular receptor,  
in a particular organism, and thereof is predicted by:

(I) providing a panel of "Biokeys", the "Biokeys"  
having a differential ability to bind the receptor in the  
presence or absence of one or more ligands, said panel  
10 therefore being able to discriminate among two or more  
different receptor conformations,

(II) screening a set of two or more reference  
substances, which are known pharmacological agonists or  
antagonists of the receptor in one or more organisms and  
15 tissues, for the ability to alter the binding of the "Biokeys"  
to the receptor, thereby obtaining a reference "fingerprint",  
for each reference substance, which is an array of descriptors,  
each descriptor defining, qualitatively or quantitatively, the  
effect of the reference compound on the binding of a Biokey  
20 panel member to the receptor.

(III) The test compound is similarly screened  
for its ability to alter the binding of the "Biokeys" to the  
receptor, thereby obtaining a test fingerprint,

(IV) the similarity of the test fingerprint to  
25 each of the reference fingerprint to each of the reference  
fingerprints is determined, and

(V) the biological activity of the test  
substance in one or more target organisms, and in one or more  
target tissues thereof, is predicted on the basis of the  
30 biological activities of the reference substances therein,  
appropriately weighted by the similarity between the test  
substance and the reference substance.

The Biokey panel of step (I) is preferably obtained  
by screening the members of a combinatorial library for the  
35 ability to bind to (a) the unliganded receptor, and (b) a  
liganded receptor. In one embodiment, a combinatorial library

is first screened against (a), and then either the whole library, or only the unliganded receptor-binding members, are screened against (b). In another embodiment, the whole library is screened against (a) and (b) simultaneously. It is also  
5 premissible to screen first against (b) and then against (a).

Preferably, the combinatorial library is an amplifiable combinatorial library, i.e., a library of nucleic acids or peptides. The members of the Biokey panel may be individual molecules, or mixtures of molecules with similar binding  
10 characteristics.

It will be appreciated that step (II) need only be performed once for a given receptor and that it is not necessary that all reference substances be fingerprinted simultaneously. Also, steps (II) and (III) may be  
15 interchanged.

In step (IV), similarity may be determined in a qualitative and subjective way, i.e., by "eyeballing" the fingerprints and judging from experience which is more similar, or in a quantitative and objective manner, using the similarity  
20 measures set forth infra.

Similarly, in step (V), the biological activity may be predicted in a qualitative and subjective way, or more quantitatively and objectively, by mathematically weighting each reference substance's activity scores by the calculated  
25 similarity of its fingerprint to the fingerprint of the test substance.

By way of example, peptides (BioKeys) that bind to the ER can be classified based on their ability to bind to the ER in the presence or absence of ER agonists. The different  
30 affinities of the peptides are due to alterations in receptor conformation following binding of an agonist. Since SERMs also uniquely alter receptor conformation, it is likely that they can affect the binding of the peptides from the different classes as well. Each agonist or SERM has associated  
35 pharmacological effects. For example, estrogen has stimulatory activity in breast and uterus, bone and the cardiovascular system. Likewise, tamoxifen is stimulatory in the uterus, bone and the cardiovascular system, but it has antagonistic effects

in the breast. The pattern of BioKey binding to the ER in response to each compound could be matched with the pharmacological effect of each compound. Additionally, a comparison between BioKey fingerprints on ER  $\alpha$  and  $\beta$  will supplement the information on agonist and antagonist activity and should be predictive of tissue specificity. New estrogen agonists and antagonists could then be screened and classified based on their BioKey binding pattern to ER  $\alpha$  and  $\beta$ , and compounds with a desired tissue-specific activity could be more readily identified.

Hypothetical Table of a "BioKey Fingerprint" for a Hypothetical Nuclear Receptor

	Compound A	Compound B	Compound C	Compound D	Compound E
BioKey 1	+	+	+	+	+
BioKey 2	+	+	+	+	-
BioKey 3	+	+	+	-	-
BioKey 4	+	+	-	-	-
BioKey 5	+	-	-	-	-

Hypothetical Table of Pharmacological Effects of Receptor Modulating Compounds

	Breast	Uterus	Bone	Cardiovascular
Compound A	+	+	+	+
Compound B	-	+	-	+
Compound C	-	+	-	-
Compound D	+	+	-	-
Compound E	+	+	-	+

For example, using the above tables, compounds with unknown pharmacological effects could be characterized by "BioKey fingerprinting" to predict their activity in various tissues. A compound X, that had a fingerprint similar to compound A, would be predicted to have pharmacological effects similar to compound A. The binding or lack of binding of a specific BioKey with the receptor could indicate activity in a specific tissue type. In the above examples, binding of BioKey 1 to the receptor in the presence of a compound could

indicate activity in the uterus. Whereas binding of BioKey 5 to the receptor in the presence of compound could indicate activity in bone.

#### Substances

5 A "substance" may be either a pure compound, or a mixture of compounds. Preferably it is at least substantially pure, that is, sufficiently pure enough to be acceptable for clinical use. If it is a mixture, then it comprises at least an effective amount (i.e., able to give rise to a detectable  
10 biological response in a biological assay) of a biologically active compound, or it comprises a substantial amount of a compound which is suspected of being biologically active and is suitable as a drug lead if so active.

#### Test substances and Drug Leads

15 A test substance comprises an effective amount of a compound, which is a member of a structural class which is generally suitable, in terms of physical characteristics (e.g., solubility), as a source of drugs and which is not known to have the pharmacological activity of interest. A drug lead is  
20 a former test substance which has either been predicted to have desirable pharmacological activity, or in fact has been shown to have such activity, and which therefore could serve effectively as a starting point for the design of analogues and derivatives which are useful as drugs. The "drug lead" may be  
25 a useful drug in its own right, or it may be a substance which is deficient as a drug because of inadequate potency or undesirable side effects. In the latter case, analogues and derivatives are sought which overcome these deficiencies. In the former case, one seeks to improve the already useful drug.

30 Such analogues and derivatives may be identified by rational drug design, or by screening of combinatorial or noncombinatorial libraries of analogues and derivatives.

Preferably, a drug lead is a compound with a molecular weight of less than 1,000, more preferably, less than 750,  
35 still more preferably, less than 600, most preferably, less than 500. Preferably, it has a computed log octanol-water

partition coefficient in the range of -4 to +14, more preferably, -2 to +7.5.

A small organic compound library is a library of compounds each of which has a molecular weight of less than 1000, and  
5 which are not peptides or nucleic acids.

SERMs from distinct structural classes may produce fingerprints unique to its class. In addition SERMs from different classes that have similar biological activities should produce similar fingerprints. Numerous SERMs that have  
10 been identified can be fingerprinted in our system. These include steroidal antiestrogens such as the ICI compounds 164,384 and 182,780, and non-steroidal compounds such as the benzothiophene derivative Raloxifene, and triphenylethylene derivatives Toremifene, Droloxifene, TAT-59 and Idoxifene. We  
15 have found that the steroidal SERMs will produce fingerprints distinct from the non-steroidal SERMs (see Example 2). Steroidal compounds such as the ICI compounds have been categorized as pure anti-estrogens, in that there is no well documented evidence of any estrogenic effects in response to  
20 these compounds. These fingerprints may be similar to the unliganded (inactive) receptor, or they may indicate that a co-repressor is bound more tightly or that a co-activator is completely inhibited from binding.

The fingerprinting system should be useful for  
25 identifying agonistic and antagonistic components from complex mixtures. The prescription drug Premarin is used for the treatment of post-menopausal symptoms. It is a complex mixture derived from the urine of pregnant mares. The active components of this mixture are not known. Fractionation of  
30 Premarin followed by fingerprinting of the individual components would indicate which of the components play an active role in modulating estrogen receptor function. It is also likely that components of Premarin interact with other nuclear receptors such as the progesterone receptor. The  
35 effect of these components could be determined as well.

#### Reference Ligands

A reference ligand is a substance which is a ligand

for a target receptor. Preferably, it is a pharmacological agonist or antagonist of a target receptor protein in one or more target tissues of a target organism. However, a reference ligand may be useful, even if it is not an agonist or antagonist, if it alters the conformation of its receptor, e.g., such that at least some Biokeys which bound the unliganded receptor do not bind as well, or bind better, the liganded receptor. Preferably, a reference ligand has a differential effect on Biokeys, so that Biokeys may be differentiated on the basis of their interaction with the receptor in the presence of the reference ligand. A reference ligand may be an agonist of one receptor and an antagonist of another. It may also be agonist of a receptor in one tissue and an antagonist of the same receptor in another tissue, or in another organism.

The reference ligand may be, but need not be, a natural ligand of the receptor.

The reference ligands may, but need not, satisfy some or all of the desiderata set forth above for test substances and drug leads.

If a test substance from one screening becomes a drug lead, and that compound, or an analogue thereof, is ultimately found to mediate the biological activity of at least one receptor in at least one tissue of at least one organism, it may be used as a reference ligand in subsequent screenings of other test substances, and in redefining the Biokey panel.

#### Reference Conformation

When a target receptor is in an unliganded state, it has a particular conformation, i.e., a particular 3-D structure. When the receptor is complexed to a ligand, the receptor's conformation changes. If the ligand is a pharmacological agonist, the new conformation is one which interacts with other components of a biological signal transduction pathway, e.g.; transcription factors, to elicit a biological response in the target tissue. If the ligand is a pharmacological antagonist, the new conformation is one in which the receptor cannot be activated by one or more agonists

which otherwise could activate that receptor.

Each of the conformations of a target receptor which is used as a binding target in a binding array is considered a reference conformation.

5           It may be that two different ligands will coincidentally cause a receptor to assume the same conformation. However, for the purpose of this invention, those will be considered different reference conformations because different ligands are involved.

10           Biokeys

For the purpose of the present invention, Biokeys are substances whose ability to bind to a target receptor in the presence or absence of one or more reference ligands for that receptor can be used to differentiate the reference ligands, and ultimately to calculate the degree of similarity between  
15 a test substance (having an assayable effect on the binding of the Biokeys to the target receptor protein) and reference substances (likewise having an assayable effect as such binding, but whose effect on biological activity of the  
20 receptor protein in target organisms and tissues of interest is also known).

Preferably, Biokeys are members of a combinatorial library, and in particular an amplifiable combinatorial library such as a peptide or nucleic acid library. The library may  
25 then be screened for binding to various receptor conformations. Biokeys need not themselves be suitable as drug leads.

Biokey Panel

For the purpose of fingerprinting the reference and test substances, a representative selection of Biokeys are  
30 collected into a panel. If only a single reference ligand is known for a receptor, the panel could include one or more representative members of each of at least two of the following binding classes:



	<u>Class</u>	<u>Binds UL-R</u>	<u>Change in Binding (Effect of Ligand)</u>
	A	+	+
	B	+	-
5	C	+	0
	D	-	0
	E	-	+

Thus classes A, B and C bind unliganded receptor (UL-R), but the ligand increases the binding of A, decreases the binding of B, and has no effect on the binding of C. Classes D and E do not bind the UL-R. The ligand causes E, but not D, to bind the receptor.

Instead of only two of the above, the panel can include representative members of three, four or all five of the classes, if Biokeys having the appropriate properties can be identified.

The above classes look at binding in only a qualitative manner. However, it would be possible to differentiate between strong and weak binders of UL-R, and between large and small changes in binding as the result of the ligand. If desired, one could draw even finer divisions, e.g.; strong vs. moderate vs. weak, etc.

If more than one ligand is available, the combinatorial possibilities are increased, and, if suitable Biokeys can be identified, the panel can be expanded appropriately.

For example, with two ligands, the following possibilities could exist

	<u>Biokey</u>	<u>UL-R</u>	<u>Ligand A</u>	<u>Ligand B</u>
	Z	+	+	+
30	Y	+	+	0
	X	+	+	-
	W	+	0	+
	V	+	0	0
	U	+	0	-
35	T	+	-	+
	S	+	-	0
	R	+	-	-
	Q	-	0	0
	P	-	+	0
40	O	-	0	+
	N	-	+	+

And one could discriminate further, e.g., for Z-1, the effect of A is greater than that of B, for Z-2, the

reverse, and for Z-3, the effects are equal.

Preferably, one, two, three, four, five or more reference ligands are used to define the Biokey panel.

It is not necessary that a particular binding class  
5 be represented by only a single Biokey. Instead, it may be represented by a mixture of two or more Biokeys, and indeed the mixture may correspond to all of the Biokeys in the Biokey library which satisfied the binding criteria for the class in question.

10 The members of the Biokey panel are chosen with a view to maximizing the discriminatory power of the panel. For example, to take an extreme case, if two members of the panel have identical binding properties, vis-a-vis, all the available reference conformations of the receptor, then one of these  
15 members is redundant. While including it in the panel does no harm, it needlessly increases the costs of the screening.

The similarity of any pair of potential panel members may be determined using the similarity measures set forth infra. The overall diversity of a given panel may be  
20 determined by computing all of the pairwise dissimilarities. For a given size panel, extracted from a given library, one may seek to maximize the overall diversity of effect on biological activity. Or one may seek to determine, for a set of binding members from a library, what is the size and composition of the  
25 subject which maximizes the ratio of the overall diversity to the number of members.

The number of panel-based descriptors in the fingerprint will normally be equal to the number of members in the panel. The optimal number of members depends on the number  
30 of reference substances, and the ability of the panel to differentiate them. The larger the number of reference substances, and the larger the number of target organisms and tissues in which the biological activity of the reference substance is to be predicted, the larger the panel should be.  
35 Typically, there will be 2, 3, 4, 5, 6, 7, 8, 9, or 10 panel members. More members may be used, but the cost of the assay increases, without necessarily providing a commensurate increase in the predictive power of the data.

### Reference substances

Reference substances are known pharmacological agonists or antagonists for the receptor in question, and have  
5 a known or ascertainable biological activity in one or more organisms and/or tissues.

Typically, for a given receptor, one, two, three, four, five or more reference substances will be fingerprinted.

### "Fingerprinting" of Test and Reference Substances

10 Each test substance will be characterized by a plurality of descriptors (the "fingerprint") by which it may be compared to reference substances.

These reference substances may be the particular reference ligands used to define the Biokey panel, but are not  
15 limited to those reference ligands. Thus, in example 1, only estradiol was used to define the five classes of peptides, but the reference substances were estradiol, estriol, tamoxifen, nafoxidine and clomiphene. The use of estradiol was not critical; the reference substances need not include any of the  
20 reference ligands used to define the BioKey panel.

The reference substances must be pharmacological agonists or antagonists in at least one organism and tissue, while the reference ligands are not so limited.

For the purpose of the present invention, a plurality  
25 of descriptors must refer to the effect of the test substance on the binding of a member of the Biokey panel to a reference conformation, e.g., unliganded receptor X, receptor X/ligand A, receptor X/ligand B, unliganded receptor Y, receptor Y/ligand C, etc. Note that in this context, the term "member"  
30 may refer to a mixture of Biokeys of the same binding class. The descriptor may be qualitative (binds vs. nonbinds; increases vs. decreases vs. no effect, etc.) or quantitative. Preferably, at least 2-10 Biokey-based descriptors are used.

The test substance may additionally be characterized  
35 by other descriptors, such as structural descriptors, known in the art. Preferably, at least 5-10 different reference substances are "fingerprinted".

The reference substances will be characterized in a similar manner to the test substances, so that their descriptors may be "paired" with the test substance descriptors in such a manner that the degree of similarity may be  
5 calculated.

When fingerprinting a given reference or test substance, it may be screened simultaneously against all panel members, or individual panel members (or subsets of panel members) may be tested separately. Also, all reference  
10 substances may be screened simultaneously against a given receptor/panel member combination, or the reference substances may be screened individually. The same is true of the screening of the test substances. The test substances may be screened after, before or simultaneously with the reference  
15 substances.

#### Descriptors

A "descriptor" (also known as a parameter, character, variable, or variate) is a numerically expressed characteristic of a compound (which may be a protein, or a protein ligand),  
20 which helps to distinguish that compound from others. A descriptor value need not be absolutely specific to a compound to be useful. The characteristics may be pure structural characteristics (as in a "structural descriptor") or they may refer to the compound's interaction with other compounds.  
25 "Paired Descriptors" are descriptors of the same property as measured in two different molecules. A "descriptor array", "list", or "set" is an array, list or set whose elements are different descriptors for the same molecule. Such an array, list or set is referred to herein as a "fingerprint".

30 A plurality of paired descriptors for two compounds may be used to calculate a similarity between the two compounds.

#### Similarity Measures

A similarity measure or coefficient quantifies the  
35 relationship between two individuals (compounds), given the values of a set of variates (descriptors) common to both.

Similarity coefficients are usually defined to take values in the range of 0 to 1.

One commonly used measure of similarity is the product moment correlation coefficient. Its correlation is unity whenever two profiles are parallel, regardless of how far apart they are in level. Two profiles may have correlation of +1 even if they are not parallel, provided that the two sets of scores are linearly related.

For binary descriptors, the simplest measure of similarity is the simple matching coefficient

$$s_{ij} = \frac{\text{number of matches}}{\text{number of comparisons}}$$

The Jaccard or Sneath coefficient modifies the simple matching coefficient by ignoring bits which in both  $i$  and  $j$  are zero, i.e., by ignoring negative matches (mutual absences). In other words, it is obtained by dividing the number of bits which are set in both descriptor bit strings, and dividing by the total number of bits set in either descriptor string. It is also called the unweighted Tanimoto coefficient.

The weighted Tanimoto coefficient for descriptors  $k$  and individuals  $i$  and  $j$  is:

$$s_{ij} = \frac{\sum_k w_k x_{ik} x_{jk}}{\sum_k w_k x_{ik} + \sum_k w_k x_{jk} - \sum_k w_k x_{ik} x_{jk}}$$

Gower has defined a general similarity coefficient which can be used for binary, qualitative, and quantitative data:

$$s_{ij} = \frac{\sum_{k=1}^p s_{ijk}}{\sum_{k=1}^p w_{ijk}} \quad \text{for individuals } i \text{ and } j \text{ and descriptor } k.$$

$w_{ijk}$  is set to 1 if the comparison is valid for variable  $k$ , and to 0 otherwise. If  $w_{ijk}=0$ , then  $s_{ijk}$  is 0. For binary data,  $w_{ijk}$  and  $s_{ijk}$  are both 0 if the variable is negative in both individuals. The  $s_{ijk}$  is positive only if the binary variable is positive for both individuals. For qualitative

data,  $s_{ijk}=1$  if the individuals are the same for the  $k$ th character, and  $s_{ijk}=0$  if they differ. For quantitative data,  $s_{ijk}=1-|X_{ik}-X_{jk}|/R_k$  where  $X_{ik}$  is the value of descriptor  $k$ , for individual  $i$ , and  $R_k$  is the total range of variable  $k$ .

5 Descriptors may be quantitative or qualitative. Quantitative descriptors may be integers or real numbers. Qualitative descriptors divide the data into categories which may be, but need not be, expressible as having relative magnitudes. Binary descriptors are a special case of  
10 qualitative descriptors, in which there are just two categories, typically representing the presence or absence of a feature. Qualitative data for which the variates have several levels may be treated like binary data with each level of a variate being regarded as a single binary variable (i.e.,  
15 an eight level variate expressed as eight bits). Or the levels may be numbered sequentially (i.e., an eight level variable expressed as three bits).

A set of  $n$ -descriptors defines an  $n$ -dimensional descriptor space; each compound for which a descriptor set is  
20 available may be said to occupy a point in descriptor space. The dissimilarity of two compounds may be expressed as a distance between the two points which they occupy in descriptor space.

A distance measure is a similarity measure which is  
25 also a metric, i.e., satisfies the conditions (i)  $d(x,y) \geq 0$ ; and  $d(x,y)=0$  if  $x=y$ ; (ii)  $d(x,y)+d(y,x)$ ; and (iii)  $d(x,z)+d(y,z) \geq d(x,y)$  (the metric or triangular inequality). Of course, the greater the distance, the less the similarity.

Distances may be calculated on the basis of any of  
30 a variety of distance measures known in the statistical arts.

The most commonly used distance measure is the Euclidean metric:

$$d_{ij} = (\sum_k (X_{ik} - X_{jk})^2)^{1/2}$$

35 It corresponds most closely to our intuitive sense of distance.

The absolute, city block, or Manhattan metric is

$$d_{ij} = \sum_k |X_{ik} - X_{jk}|$$

Its rationale is that if the variables have scale units of equal value, the entities should have the same distance whether two units apart on each of two variables, or one unit apart on one and three on the other.

The "cosine theta" distance is the cosine of the angle between the vector from the origin to point  $X_{ik}$  and the vector from the origin to point  $X_{jk}$ .

A generalized distance measure is the Minkowski metric:

$$d_{ij} = (\sum_k |X_{ik} - X_{jk}|^r)^{1/r}$$

which is a Euclidean metric for  $r=2$  and a city block metric for  $r=1$ .

The Mahalanobis distance measure ( $D^2$ ) is of the form

$$d_{ij} = (X_i - X_j)' \Sigma^{-1} (X_i - X_j)$$

where  $\Sigma$  is the pooled-within-groups variance-covariance matrix, and  $X_i$  and  $X_j$  are the vectors of scores for entities  $i$  and  $j$ . The Mahalanobis distance allows for correlations between variables; if the variables are uncorrelated,  $D^2$  is equivalent to Euclidean distance measured using standard variables.

The Canberra metric, given below, has the advantage of being unaffected by the range of the variable:

$$d(i,j) = \sum_k (|X_{jk} - X_{ik}|) / (X_{ik} + X_{jk}).$$

A modified form, which accommodates negative states, is

$$d(i,j) = \sum_k (|X_{jk} - X_{ik}| / (|X_{ik}| + |X_{jk}|)).$$

The Calhoun distance uses only rank orders; for molecules  $i$  and  $j$ , the distance is the proportion of the entire set (excluding  $i$  and  $j$ ) that have descriptor states intermediate between that for  $i$  and that for  $j$  for one or more of the descriptors  $k$ .

A distance measure may be transformed into a similarity measure by any of a variety of transformations that

convert a non-negative number to the range 0..1, e.g.,

$$S_{ij}=1/(1+d_{ij})$$

A similarity measure may be converted into a distance by, e.g.,  $d_{ij} = 1-s_{ij}$ .

- 5 If there is a theoretical maximum distance ( $d_{tmax}$ ), based on the theoretically possible ranges for each of the component descriptors, the similarity may be expressed as

$$S_{ij}=1-(d_{ij}/d_{tmax})$$

- Alternatively, one may calculate the distances  
10 between all pairs, and then use the actual maximum distance ( $d_{amax}$ ):

$$S_{ij}=1-(d_{ij}/d_{amax})$$

- Instead of using the ratio of the actual distance to the actual or theoretical maximum distance, one may express  $s_{ij}$   
15 as the fraction of the pairs for which the distance is greater than or equal to  $d_{ij}$ . This is a measure of relative similarity.

Descriptors may be weighted (or otherwise transformed) for any of several reasons, including:

- 20 (a) to reflect the perceived value of the descriptor for determining whether two proteins will be modulated by structurally similar drugs;
- 25 (b) to reflect the perceived reliability of the descriptor data;
- 30 (c) to correct for differences in scale between descriptors, so that a descriptor does not dominate a similarity or distance calculation merely because its values are of higher magnitude or are spread over a greater range; and
- (d) to correct for correlations between descriptors.

- The raw descriptor values may be, but need not be,  
35 transformed prior to use in calculating distances. Typical transformations are (a) presence (1)/absence (0), (b)  $\ln(x+1)$ , (c) frequency in sample, (d) root, and (e) relative range, i.e.,  $(\text{value}-\text{min})/(\text{max}-\text{min})$ .



The raw descriptor values may be standardized (normalized) to have zero mean ( $x' = x - \mu_x$ ) and/or unit variance ( $x' = x / \sigma_x$ ), possibly both ( $x' = (x - \mu_x) / \sigma_x$ ) or they be standardized (unitized) to fall into the range 0 to 1.

5           Descriptor weights may be adjusted empirically on the basis of specially designed test sets. A training set of proteins is identified. Descriptors are evaluated for each protein in the set. A training set of compounds, including  
10           are also tested against each compound in the set. These compounds are chosen so that, for any protein in the set, there is at least one compound which is an agonist or antagonist for it. A neural net, with the descriptor weights as inputs, is used to predict the activity of each compound against each protein, using the calculated protein similarities. For  
15           example, it will calculate the similarity of protein x to all other proteins, then treat the activities of the compounds against the other proteins as "knowns" and use it to predict the activity of the compounds against protein x. This is done repeatedly, with each protein taking on the role of protein x,  
20           in turn.

          The coefficient of variation may be useful in comparing descriptors; it is the standard deviation divided by the mean. If there is no information available about the ultimate significance of a descriptor, one may give a greater  
25           weight to descriptors which have a larger CV and hence a more uniform distribution.

          It must be emphasized that we do not require use of weighted descriptors, let alone of any particular method of deriving weights.

30           It is likely that some degree of correlation will exist among the descriptors. Standard mathematical methods, such as cluster analysis, principal components analysis, or partial least squares analysis, may be used to determine which descriptors are strongly correlated and to replace them with  
35           a new descriptor which is a weighted sum of the original correlated descriptors. One may alternatively choose (perhaps randomly) one of each pair of highly correlated descriptors and simply prune it, thereby reducing the amount of data which must

be collected.

One way of correcting for correlation among the descriptors is for each descriptor  $m$ , calculate the average of its squared correlation coefficients with all descriptors  $n$  (including  $m=n$ , for which the coefficient is necessarily unity), and subtract this number from one to obtain a weight representing the fraction of the variation in descriptor  $m$  which is not explained by the "average" descriptor  $n$ . With this "average  $r^2$ " method, if we have four descriptors, and two are perfectly correlated to each other, and the descriptors are otherwise completely uncorrelated, the correlated descriptors will have weights of 0.5 each, and the other two will have weights of 1.0 each.

The diversity of a set of compounds, as measured by a set of descriptors, may be calculated in several ways.

A purely geometric method involves assuming that each compound sweeps out a hypersphere in descriptor space, the hypersphere having a radius known as the similarity radius. The total hypervolume in descriptor space of points within a unit similarly radius of one or more of the compounds is calculated. This is compared to the hypervolume achievable if none of hypersphere's overlap; i.e., to  $n$  \* volume of a single hypersphere, where  $n$  is the number of compounds in the set. The swept hypervolume may be determined exactly, or by Monte Carlo methods. The ratio of the swept hypervolume to the maximum hypervolume is a measure of compound set diversity, ranging from 1 (maximum) to  $1/n$  (minimum).

Another approach is to calculate all of the pairwise distances between compounds in descriptor space. The mean distance is a measure of diversity. If desired, this can be scaled by calculating the ratio of the mean distance to the maximum theoretical distance.

A third approach is to apply cluster analysis to the set of compounds. The method used should be one which does not set the number of clusters arbitrarily, but rather decides the number based on some goodness-of-fit criterion. The resulting number of cluster is a measure of diversity, as is the ratio of the number of clusters to the number of compounds.

One may calculate a measure of disorder for a descriptor as

$$H(k) = - \sum_{g=1}^{m_k} P_{kg} \ln P_{kg}$$

where  $m_k$  is the number of different states in descriptor  $k$ , and  $P_{kg}$  is the observed proportion of individuals exhibiting state  $g$  for descriptor  $k$ . For uncorrelated descriptors, the sum of  $H(k)$  for all  $k$  is a measure of overall diversity.

Standard techniques may be used to correct for correlation.

#### Target Receptor

The target receptor may be a naturally occurring substance, or a subunit or domain thereof, from any natural source, including a virus, a microorganism (including bacterial, fungi, algae, and protozoa), an invertebrate (including insects and worms), or the normal or cancerous cells of a vertebrate (especially a mammal, bird or fish and, among mammals, particularly humans, apes, monkeys, cows, pigs, goats, llamas, sheep, rats, mice, rabbits, guinea pigs, cats and dogs). (Usually it is a protein; it may be a nucleic acid. References to proteins apply, mutatis mutandis, to nucleic acids, lipids, carbohydrates and other macromolecules which can act as receptors.) Alternatively, the receptor protein may be a modified form of a natural receptor. Modifications may be introduced to facilitate the labeling or immobilization of the target receptor, or to alter its biological activity (An inhibitor of a mutant receptor may be useful to selectively inhibit an undesired activity of the mutant receptor and leave other activities substantially intact). In the case of a protein, modifications include mutation (substitution, insertion or deletion of a genetically encoded amino acid) and derivatization (including glycosylation, phosphorylation, and lipidation).

A target receptor may be, inter alia, a glyco-, lipo-, phospho-, or metalloprotein. It may be a nuclear, cytoplasmic, membrane, or secreted protein. It may, but need not, be an enzyme.

The target receptor, instead of being a protein, may be a macromolecular nucleic acid, lipid or carbohydrate. If a nucleic acid, it may be a ribo- or a deoxribonucleic acid, and it may be single or double stranded. It may, but need not, have enzymatic activity.

The target receptor need not be a single macromolecule, rather, it may be a complex of a macromolecule with one or more additional molecules, especially macromolecules. Examples includes ribosomes (RNA:protein complexes), polysomes (mRNA:ribosome complexes), and chromatin (DNA:protein complexes). For use of polysomes as binding molecules (or as display systems), see Kawasaki, USP 5,643,768 and 5,658,754; Gersuk, et al., Biochem. Biophys. Res. Comm. 232:578 (1997); Mattheakis, et al., Proc. Nat. Acad. Sci. USA, 91:9022-6 (1994).

The known binding partners (if any) of the target receptor may be, inter alia, proteins, oligo- or polypeptides, nucleic acids, carbohydrates, lipids, or small organic or inorganic molecules or ions.

The functional groups of the receptor which participate in the ligand-binding interactions together form the ligand binding site, or paratope, of the receptor. Similarly, the functional groups of the ligand which participate in these interactions together form the epitope of the ligand.

In the case of a protein, the binding sites are typically relatively small surface patches. The binding characteristics of the protein may often be altered by local modifications at these sites, without denaturing the protein.

While it is possible for a chemical reaction to occur between a functional group on a receptor and one on a ligand, resulting in a covalent bond, receptor protein-ligand binding normally occurs as a result of the aggregate effects of several noncovalent interactions. Electrostatic interactions include salt bridges, hydrogen bonds, and van der Waals forces.

What is called the hydrophobic interaction is actually the absence of hydrogen bonding between nonpolar groups and water, rather than a favorable interaction between

the nonpolar groups themselves. Hydrophobic interactions are important in stabilizing the conformation of a receptor protein and thus indirectly affect ligand binding, although hydrophobic residues are usually buried and thus not part of the binding site.

The receptor may have more than one paratope and they may be the same or different. Different paratopes may interact with epitopes of different binding partners. An individual paratope may be specific to a particular binding partner, or it may interact with several different binding partners. A receptor can bind a particular binding partner through several different binding sites. The binding sites may be continuous or discontinuous (e.g., vis-a-vis the primary sequence of a receptor protein).

A list of agonists, antagonists, radioligands and effectors for many different receptors appears in Appendix I of King, Medicinal Chemistry: Principles and Practice, pp. 290-294 (Royal Soc'y Chem. 1994). Appendix II lists blockers for various ion channels (which are another special type of receptor). Some receptors, and their agonists and/or antagonists, are listed in Table A.

Any nuclear receptor, such as receptors for progestins, androgens, glucocorticoids, thyroid hormones, retinoids, vitamin D3 and mineralocorticoids could be used in this fingerprinting system. Affinity selection of peptide libraries could be used to identify peptide sequences that bind in the presence or absence of agonist as described above. The peptides could then be used in the manner described above to classify and characterize modulators of the receptor's activity. As described above, components of Premarin are likely to interact with the progesterone receptor. A system for fingerprinting the progesterone receptor may be developed to test for active components of Premarin.

As an example of a non-protein receptor, we cite DNA. DNA can undergo conformational changes when it is bound for example, by a transcription factor or small molecule. For example, the antitumor agent cisplatin binds to and alters the structure of DNA. The altered structure attracts a cellular

- protein containing an HMG box (high mobility group). The protein is believed to sterically block the repair of the cisplatin lesion on the DNA and contribute to the effectiveness of cisplatin in the treatment of certain types of cancer.
- 5 BioKeys could be identified that bind specifically to DNA in certain conformations. These Bikeys could be used to identify conformational changes that take place in the DNA upon binding of a small molecule or protein.

#### Target Organism

- 10 A purpose of the present invention is to predict the biological activity in one or more target tissues, as hereafter defined, of a target organism.

The target organism may be a plant, animal, or microorganism.

- 15 In the case of a plant, it may be an economic plant, in which case the drug may be intended to increase the disease, weather or pest resistance, alter the growth characteristics, or otherwise improve the useful characteristics or mute undesirable characteristics of the plant. Or it may be a weed,
- 20 in which case the drug may be intended to kill or otherwise inhibit the growth of the plant, or to alter its characteristics to convert it from a weed to an economic plant. The plant may be a tree, shrub, crop, grass, etc. The plant may be an algae (which are in some cases also microorganisms),
- 25 or a vascular plant, especially gymnosperms (particularly conifers) and angiosperms. Angiosperms may be monocots or dicots. The plants of greatest interest are rice, wheat, corn, alfalfa, soybeans, potatoes, peanuts, tomatoes, melons, apples, pears, plums, pineapples, fir, spruce, pine, cedar, and oak.

- 30 If the target organism is a microorganism, it may be algae, bacteria, fungi, or a virus (although the biological activity of a virus must be determined in a virus-infected cell). The microorganism may be human or other animal or plant pathogen, or it may be nonpathogenic. It may be a soil or
- 35 water organism, or one which normally lives inside other living things.

If the target organism is an animal, it may be a

vertebrate or a nonvertebrate animal. Nonvertebrate animals are chiefly of interest when they act as pathogens or parasites, and the drugs are intended to act as a biocidal or biostatic agents. Nonvertebrate animals of interest include  
5 worms, mollusks, and arthropods.

The target organism may also be a vertebrate animal, i.e., a mammal, bird, reptile, fish or amphibian. Among mammals, the target animal preferably belongs to the order Primata (humans, apes and monkeys), Artiodactyla (e.g., cows,  
10 pigs, sheep, goats, horses), Rodenta (e.g., mice, rats) Lagomorpha (e.g., rabbits, hares), or Carnivora (e.g., cats, dogs). Among birds, the target animals are preferably of the orders Anseriformes (e.g., ducks, geese, swans) or Galliformes (e.g., quails, grouse, pheasants, turkeys and chickens). Among  
15 fish, the target animal is preferably of the order Clupeiformes (e.g., sardines, shad, anchovies, whitefish, salmon).

#### Target Tissues

The term "target tissue" refers to any whole animal, physiological system, whole organ, part of organ, miscellaneous  
20 tissue, cell, or cell component (e.g., the cell membrane) of a target animal in which biological activity may be measured.

Routinely in mammals one would choose to compare and contrast the biological impact on virtually any and all tissues which express the subject receptor protein. The main tissues  
25 to use are: brain, heart, lung, kidney, liver, pancreas, skin, intestines, adrenal glands, breast, prostate, vasculature, retina, cornea, thyroid gland, parathyroid glands, thymus, bone marrow etc.

Another classification would be by cell type: B  
30 cells, T cells, macrophages, neutrophils, eosinophils, mast cells, platelets, megakaryocytes, erythrocytes, bone marrow stromal cells, fibroblasts, neurons, astrocytes, neuroglia, microglia, epithelial cells (from any organ, e.g. skin, breast, prostate, lung, intestines etc), cardiac muscle cells, smooth  
35 muscle cells, striated muscle cells, osteoblasts, osteocytes, chondroblasts, chondrocytes, keratinocytes, melanocytes, etc.

The "target tissues" include those set forth in Table

B. Of course, in the case of a unicellular organism, there is no distinction between the "target organism" and the "target tissue".

#### In Vitro vs. In Vivo Assays

5       The term "in vivo" is descriptive of an event, such as binding or enzymatic action, which occurs within a living organism. The organism in question may, however, be genetically modified. The term "in vitro" refers to an event  
10       which occurs outside a living organism. Parts of an organism (e.g., a membrane, or an isolated biochemical) are used, together with artificial substrates and/or conditions. For the purpose of the present invention, the term in vitro excludes events occurring inside or on an intact cell, whether of a unicellular or multicellular organism.

15       In vivo assays include both cell-based assays, and organismic assays. The term cell-based assays includes both assays on unicellular organisms, and assays on isolated cells or cell cultures derived from multicellular organisms. The cell cultures may be mixed, provided that they are not  
20       organized into tissues or organs. The term organismic assay refers to assays on whole multicellular organisms, and assays on isolated organs or tissues of such organisms.

#### Biological Assays

25       While a major purpose of the invention is to minimize the need for biological assays, they cannot be altogether avoided. In order to predict the biological activity of a substance, one must know the biological activities of a reasonable number of reference substances.

30       A biological assay measures or detects a biological response of a biological entity to a substance. The present invention is concerned with responses which are, at least in part, mediated by a receptor.

35       The biological entity may be a whole organism, an isolated organ or tissue, freshly isolated cells, an immortalized cell line, or a subcellular component (such as a



membrane; this term should not be construed as including an isolated receptor). The entity may be, or may be derived from, an organism which occurs in nature, or which is modified in some way. Modifications may be genetic (including radiation and chemical mutants, and genetic engineering) or somatic (e.g., surgical, chemical, etc.). In the case of a multicellular entity, the modifications may affect some or all cells. The entity need not be the target organism, or a derivative thereof, if there is a reasonable correlation between bioassay activity in the assay entity and biological activity in the target organism.

The entity is placed in a particular environment, which may be more or less natural. For example, a culture medium may, but need not, contain serum or serum substitutes, and it may, but need not, include a support matrix of some kind, it may be still, or agitated. It may contain particular biological or chemical agents, or have particular physical parameters (e.g., temperature), that are intended to nourish or challenge the biological entity.

There must also be a detectable biological marker for the response. At the cellular level, the most common markers are cell survival and proliferation, cell behavior (clustering, motility), cell morphology (shape, color), and biochemical activity (overall DNA synthesis, overall protein synthesis, and specific metabolic activities, such as utilization of particular nutrients, e.g., consumption of oxygen, production of CO<sub>2</sub>, production of organic acids, uptake or discharge of ions).

The direct signal produced by the biological marker may be transformed by a signal producing system into a different signal which is more observable, for example, a fluorescent or colorimetric signal.

The entity, environment, marker and signal producing system are chosen to achieve a clinically acceptable level of sensitivity, specificity and accuracy.

Reference substances should be tested in the appropriate assays relevant to the tissue distribution of the targeted receptor. For instance, for the estrogen receptor

which is expressed in breast epithelium, liver mesenchymal cells, osteoclasts and uterine epithelium (among others) appropriate assays would include, among others, breast and uterine epithelial cell proliferation, osteoclast apoptosis, and hepatocyte production of lipids such as triglycerides and cholesterol and lipoproteins such as high density lipoproteins and low density lipoproteins.

If one were to utilize the androgen receptor which is expressed in, among others, prostate epithelium, hepatocytes, striated muscle cells, then one would might chose to carry out assays of the reference substance set for, among others, prostate hypertrophy, hyperplasia or prostate epithelial cell proliferation, muscle cell hyperplasia or hypertrophy and heptotoxicity etc.

As another example, if one were to utilize the beta-2-adrenergic receptor, which is expressed in, among others, the heart, brain and peripheral vasculature, then one may chose to test reference substances in cardiac function assays (such as cardiac rate and eletrocardiographic changes), assays for their impact on blood pressure and assays to evaluate their impact on neuronal activity within the central nervous system.

#### Preliminary Screening Assays

The invention contemplates three occasions for preliminary screening:

- (a) screening for potential "BioKeys", using a known receptor and one or more known pharmacological modulators of the receptor (see General Method step (I)),
- (b) screening reference compounds, having a known receptor-mediated bioactivity using a known receptor and an established BioKey panel, to obtain reference fingerprints (see General Method, step (II), and
- (c) screening test compounds for their ability to alter the binding of a panel of BioKeys to the receptor, thereby obtaining a test fingerprint (see General Method, step (III)).

The same or different screening methods may be used on each occasion.

Preliminary, screening assays will typically be either in vitro (cell-free) assays (for binding to an immobilized  
5 receptor) or cell-based assays (for alterations in the phenotype of the cell). They will not involve screening of whole multicellular organisms, or isolated organs. The comments on biological assays apply mutatis mutandis to preliminary screening cell-based assays.

10 In a preferred cell-based assay, the receptor is functionally connected to a signal (biological marker) producing system, which may be endogenous or exogenous to the cell.

#### "Zero-Hybrid" Systems

15 In these systems, the binding of a peptide to the target protein results in a screenable or selectable phenotypic change, without resort to fusing the target protein (or a ligand binding moiety thereof) to an endogenous protein. It may be that the target protein is endogenous to the host cell,  
20 or is substantially identical to an endogenous receptor so that it can take advantage of the latter's native signal transduction pathway. Or sufficient elements of the signal transduction pathway normally associated with the target protein may be engineered into the cell so that the cell  
25 signals binding to the target protein.

#### "One-Hybrid" Systems

In these systems, a chimera receptor, a hybrid of the target protein and an endogenous receptor, is used. The chimeric receptor has the ligand binding characteristics of the  
30 target protein and the signal transduction characteristics of the endogenous receptor. Thus, the normal signal transduction pathway of the endogenous receptor is subverted.

Preferably, the endogenous receptor is inactivated, or the conditions of the assay avoid activation of the endogenous  
35 receptor, to improve the signal-to-noise ratio.

See Fowlkes USP 5,789,184 for a yeast system.

Another type of "one-hybrid" system combines a peptide: DNA-binding domain fusion with an unfused target receptor that possesses an activation domain.

#### "Two-Hybrid" System

5 In a preferred embodiment, the cell-based assay is a two hybrid system. One member of a peptide ligand:receptor binding pair is expressed as a fusion to a DNA-binding domain (DBD) from a transcription factor (this fusion protein is called the "bait"), and the other is expressed as a fusion to a  
10 transactivation domain (TAD) (this fusion protein is called the "fish", the "prey", or the "catch"). The transactivation domain should be complementary to the DNA-binding domain, i.e., it should interact with the latter so as to activate transcription of a specially designed reporter gene that  
15 carries a binding site for the DNA-binding domain. Naturally, the two fusion proteins must likewise be complementary.

This complementarity may be achieved by use of the complementary and separable DNA-binding and transcriptional activator domains of a single transcriptional activator  
20 protein, or one may use complementary domains derived from different proteins. The domains may be identical to the native domains, or mutants thereof. The assay members may be fused directly to the DBD or TAD, or fused through an intermediated linker.

25 The target DNA operator may be the native operator sequence, or a mutant operator. Mutations in the operator may be coordinated with mutations in the DBD and the TAD. An example of a suitable transcription activation system is one comprising the DNA-binding domain from the bacterial repressor  
30 LexA and the activation domain from the yeast transcription factor Gal4, with the reporter gene operably linked to the LexA operator.

It is not necessary to employ the intact target receptor; just the ligand-binding moiety is sufficient.

35 The two fusion proteins may be expressed from the same or different vectors. Likewise, the activatable reporter gene may be expressed from the same vector as either fusion protein (or

both proteins), or from a third vector.

Potential DNA-binding domains include Gal4, LexA, and mutant domains substantially identical to the above.

Potential activation domains include E. coli B42, Gal4  
5 activation domain II, and HSV VP16, and mutant domains substantially identical to the above.

Potential operators include the native operators for the desired activation domain, and mutant domains substantially identical to the native operator.

10 The fusion proteins may comprise nuclear localization signals.

The assay system will include a signal producing system, too. The first element of this system is a reporter gene operably linked to an operator responsive to the DBD and TAD  
15 of choice. The expression of this reporter gene will result, directly or indirectly, in a selectable or screenable phenotype (the signal). The signal producing system may include, besides the reporter gene, additional genetic or biochemical elements which cooperate in the production of the signal. Such an  
20 element could be, for example, a selective agent in the cell growth medium. There may be more than one signal producing system, and the system may include more than one reporter gene.

The sensitivity of the system may be adjusted by, e.g., use of competitive inhibitors of any step in the activation or  
25 signal production process, increasing or decreasing the number of operators, using a stronger or weaker DBD or TAD, etc.

When the signal is the death or survival of the cell in question, or proliferation or nonproliferation of the cell in question, the assay is said to be a selection. When the signal  
30 merely results in a detectable phenotype by which the signalling cell may be differentiated from the same cell in a nonsignalling state (either way being a living cell), the assay is a screen. However, the term "screening assay" may be used in a broader sense to include a selection. When the narrower  
35 sense is intended, we will use the term "nonselective screen".

Various screening and selection systems are discussed in Ladner, USP 5,198,346.

Screening and selection may be for or against the peptide:

target protein or compound:target protein interaction.

Preferred assay cells are Microbial (bacterial, yeast, algal, protozoal), invertebrate (esp. mammalian, particularly human). The best developed two-hybrid assays are yeast and  
5 mammalian systems.

Normally, two hybrid assays are used to determine whether a protein X and a protein Y interact, by virtue of their ability to reconstitute the interaction of the DBD and the TAD. However, augmented two-hybrid assays have been used to detect  
10 interactions that depend on a third, non-protein ligand.

For more guidance on two-hybrid assays, see Brent and Finley, Jr., *Ann. Rev. Genet.*, 31:663-704 (1997); Fremont-Racine, et al., *Nature Genetics*, 277-281 (16 July 1997); Allen, et al., *TIBS*, 511-16 (Dec. 1995); LeCrenier, et al., *BioEssays*,  
15 20:1-6 (1998); Xu, et al., *Proc. Nat. Acad. sci. (USA)*, 94:12473-8 (Nov. 1992); Esotak, et al., *Mol. Cell. Biol.*, 15:5820-9 (1995); Yang, et al., *Nucleic Acids Res.*, 23:1152-6 (1995); Bendixen, et al., *Nucleic Acids Res.*, 22:1778-9 (1994); Fuller, et al., *BioTechniques*, 25:85-92 (July 1998); Cohen, et al., *PNAS (USA)* 95:14272-7 (1998); Kolonin and Finley, Jr., *PNAS (USA)* 95:14266-71 (1998). See also Vasavada, et al., *PNAS (USA)*, 88:10686-90 (1991) (contingent replication assay), and Rehrauer, et al., *J. Biol. Chem.*, 271:23865-73 (1996) (LexA repressor cleavage assay).

## 25 "Substantially Identical"

A mutant protein is substantially identical to a reference protein if (a) it has at least 10% of a specific binding activity or a non-nutritional biological activity of the reference protein, and (b) is at least 50% identical in amino  
30 acid sequence to the reference protein.

Percentage amino acid identity is determined by aligning the mutant and reference sequences according to a rigorous dynamic programming algorithm which globally aligns their sequences to maximize their similarity, the similarity being  
35 scored as the sum of scores for each aligned pair according to an unbiased PAM250 matrix, and a penalty for each internal gap of -12 for the first null of the gap and -4 for each additional

null of the same gap. The percentage identity is the number of matches expressed as a percentage of the adjusted (i.e., counting inserted nulls) of the reference sequence.

5 A mutant DNA sequence is substantially identical to a reference DNA sequence if they are structural sequences, and encoding mutant and reference proteins which are substantially identical as described above.

If instead they are regulatory sequences, they are substantially identical if the mutant sequence has at least 10%  
10 of the regulatory activity of the reference sequence, and is at least 50% identical in nucleotide sequence to the reference sequence. Percentage identity is determined as for proteins except that matches are scored +5, mismatches -4, the gap open penalty is -12, and the gap extension penalty (per null) is -4.

15 Preferably, sequence which are substantially identical exceed the minimum identity of 50% are, e.g., 51%, 66%, 75%, 80%, 85%, 90%, 95% or 99% identical in sequence.

DNA sequences may also be considered "substantially identical" if they hybridize to each other under stringent  
20 conditions, i.e., conditions at which the  $T_m$  of the heteroduplex of the one strand of the mutant DNA and the more complementary strand of the reference DNA is not in excess of 10°C. less than the  $T_m$  of the reference DNA homoduplex. Typically this will correspond to a percentage identity of 85-  
25 90%.

#### Combinatorial Libraries

The term "library" generally refers to a collection of chemical or biological entities which are related in origin, structure, and/or function, and which can be screened  
30 simultaneously for a property of interest.

The term "combinatorial library" refers to a library in which the individual members are either systematic or random combinations of a limited set of basic elements, the properties of each member being dependent on the choice and location of  
35 the elements incorporated into it. Typically, the members of the library are at least capable of being screened simultaneously. Randomization may be complete or partial; some

positions may be randomized and others predetermined, and at random positions, the choices may be limited in a predetermined manner. The members of a combinatorial library may be oligomers or polymers of some kind, in which the variation occurs through the choice of monomeric building block at one or more positions of the oligomer or polymer, and possibly in terms of the connecting linkage, or the length of the oligomer or polymer, too. Or the members may be nonoligomeric molecules with a standard core structure, like the 1,4-benzodiazepine structure, with the variation being introduced by the choice of substituents at particular variable sites on the core structure. Or the members may be nonoligomeric molecules assembled like a jigsaw puzzle, but wherein each piece has both one or more variable moieties (contributing to library diversity) and one or more constant moieties (providing the functionalities for coupling the piece in question to other pieces).

The ability of one or more members of such a library to recognize a target molecule is termed "Combinatorial Recognition". In a "simple combinatorial library", all of the members belong to the same class of compounds (e.g., peptides) and can be synthesized simultaneously. A "composite combinatorial library" is a mixture of two or more simple libraries, e.g., DNAs and peptides, or benzodiazepine and carbamates. The number of component simple libraries in a composite library will, of course, normally be smaller than the average number of members in each simple library, as otherwise the advantage of a library over individual synthesis is small.

#### Oligonucleotide Libraries

An oligonucleotide library is a combinatorial library, at least some of whose members are single-stranded oligonucleotides having three or more nucleotides connected by phosphodiester or analogous bonds. The oligonucleotides may be linear, cyclic or branched, and may include non-nucleic acid moieties. The nucleotides are not limited to the nucleotides normally found in DNA or RNA. For examples of nucleotides modified to increase nuclease resistance and chemical stability



of aptamers, see Chart 1 in Osborne and Ellington, Chem. Rev., 97: 349-70 (1997). For screening of RNA, see Ellington and Szostak, Nature, 346: 818-22 (1990).

There is no formal minimum or maximum size for these  
5 oligonucleotides. However, the number of conformations which  
an oligonucleotide can assume increases exponentially with its  
length in bases. Hence, a longer oligonucleotide is more  
likely to be able to fold to adapt itself to a protein surface.  
On the other hand, while very long molecules can be synthesized  
10 and screened, unless they provide a much superior affinity to  
that of shorter molecules, they are not likely to be found in  
the selected population, for the reasons explained by Osborne  
and Ellington (1997). Hence, the libraries of the present  
invention are preferably composed of oligonucleotides having  
15 a length of 3 to 100 bases, more preferably 15 to 35 bases.  
The oligonucleotides in a given library may be of the same or  
of different lengths.

Oligonucleotide libraries have the advantage that  
libraries of very high diversity (e.g.,  $10^{15}$ ) are feasible, and  
20 binding molecules are readily amplified in vitro by polymerase  
chain reaction (PCR). Moreover, nucleic acid molecules can  
have very high specificity and affinity to targets.

In a preferred embodiment, this invention prepares  
and screens oligonucleotide libraries by the SELEX method, as  
25 described in King and Famulok, Molec. Biol. Repts., 20: 97-107  
(1994); L. Gold, C. Tuerk. Methods of producing nucleic acid  
ligands, US#5595877; Oliphant et al. Gene 44:177 (1986).

The term "aptamer" is conferred on those  
oligonucleotides which bind the target protein. Such aptamers  
30 may be used to characterize the target protein, both directly  
(through identification of the aptamer and the points of  
contact between the aptamer and the protein) and indirectly (by  
use of the aptamer as a ligand to modify the chemical  
reactivity of the protein).

### 35 Peptide Library

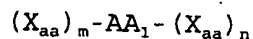
A peptide library is a combinatorial library, at  
least some of whose members are peptides having three or more

amino acids connected via peptide bonds. Preferably, they are at least five, six, seven or eight amino acids in length. Preferably, they are composed of less than 50, more preferably less than 20 amino acids.

- 5           The peptides may be linear, branched, or cyclic, and may include nonpeptidyl moieties. The amino acids are not limited to the naturally occurring amino acids.

A biased peptide library is one in which one or more (but not all) residues of the peptides are constant residues.

- 10   The individual members are referred to as peptide ligands (PL). In one embodiment, an internal residue is constant, so that the peptide sequence may be written as



- Where Xaa is either any naturally occurring amino acid,  
15   or any amino acid except cysteine, m and n are chosen independently from the range of 2 to 20, the Xaa may be the same or different, and AA<sub>1</sub> is the same naturally occurring amino acid for all peptides in the library but may be any amino acid. Preferably, m and n are chosen independently from the  
20   range of 4 to 9.

- Preferably, AA<sub>1</sub> is located at or near the center of the peptide. More specifically, it is desirable that m and n are not different by more than 2; more preferably m and n are equal. Even if the chosen AA<sub>1</sub> is required (or at least  
25   permissive) of the target protein (TP) binding activity, one may need particular flanking residues to assure that it is properly positioned. If AA<sub>1</sub> is more or less centrally located, the library presents numerous alternative choices for the flanking residues. If AA<sub>1</sub> is at an end, this flexibility is  
30   diminished.

- The most preferred libraries are those in which AA<sub>1</sub> is tryptophan, proline or tyrosine. Second most preferred are those in which AA<sub>1</sub> is phenylalanine, histidine, arginine, aspartate, leucine or isoleucine. Third most preferred are  
35   those in which AA<sub>1</sub> is asparagine, serine, alanine or methionine. The least preferred choices are cysteine and glycine. These preferences are based on evaluation of the results of screening random peptide libraries for binding to

many different TPs.

Ligands that bind to functional domains tend to have both constant as well as unique features. Therefore, by using "biased" peptide libraries, one can ease the burden of finding  
5 ligands. Either "biased" or "unbiased" libraries may be screened to identify "BioKey" peptides for use in developing reactivity descriptors, and, optionally, peptide aptamer descriptors and additional drug leads.

#### Studies of Orphan Receptors

10 Orphan receptors have been identified by virtue of their sequence similarity to known non-orphan receptors, however, by definition, they do not have known natural ligands.

The first step in seeking to predict an orphan receptor-mediated biological activity of a compound is to  
15 identify at least one pharmacological agonist or antagonist of the orphan receptor. (Once such a compound is identified, the receptor is not longer strictly speaking an "orphan".) This ligand, which need not be a natural ligand of the receptor, is then used as a reference ligand to define a Biokey panel, etc.

20 To identify an agonist or antagonist, a combinatorial library is first screened for members which bind the receptor. Preferably, at least five, more preferably at least ten, distinct members are identified. Preferably, it should be demonstrable from competition experiments that more than one  
25 binding site is involved.

Compounds are then screened for the ability to inhibit the binding of one or more of the aforementioned library members to the orphan receptor. Those which do so are likely to have altered the receptor conformation. These  
30 putative ligands are then screened for agonist or antagonist activity. The biological activities examined preferably include the activities native to those of the cognate receptors by reference to which the orphan receptors were originally identified. They also preferably include assays for cell  
35 proliferation for each cell type in which said orphan receptor is known (by detection of the receptor or its corresponding mRNA) to be expressed.

The screened compounds may be small organic compounds, such as compounds from a suitable combinatorial or noncombinatorial library, or they may come from natural sources, such as serum, urine, cerebrospinal fluid, lymphatic fluid, or tissue extracts, which might harbor the natural ligand. Optionally, these natural source materials may be fractionated by conventional methods, and each fraction tested. The compounds may be known agonists or antagonists (or analogues thereof) of the cognate receptor, but need not be.

10        Small Organic Compound Library

The small organic compound library ("compound library", for short) is a combinatorial library whose members are suitable for use as drugs if, indeed, they have the ability to mediate a biological activity of the target protein.

15        Peptides have certain disadvantages as drugs. These include susceptibility to degradation by serum proteases, and difficulty in penetrating cell membranes. Preferably, all or most of the compounds of the compound library avoid, or at least do not suffer to the same degree, one or more of the pharmaceutical disadvantages of peptides.

20        In designing a compound library, it is helpful to bear in mind the methods of molecular modification typically used to obtain new drugs. Three basic kinds of modification may be identified: disjunction, in which a lead drug is simplified to identify its component pharmacophoric moieties; conjunction, in which two or more known pharmacophoric moieties, which may be the same or different, are associated, covalently or noncovalently, to form a new drug; and alteration, in which one moiety is replaced by another which may be similar or different, but which is not in effect a disjunction or conjunction. The use of the terms "disjunction", "conjunction" and "alteration" is intended only to connote the structural relationship of the end product to the original leads, and not how the new drugs are actually synthesized, although it is possible that the two are the same.

35        The process of disjunction is illustrated by the evolution of neostigmine (1931) and edrophonium (1952) from

physostigmine (1925). Subsequent conjunction is illustrated by demecarium (1956) and ambenonium (1956).

Alterations may modify the size, polarity, or electron distribution of an original moiety. Alterations include ring closing or opening, formation of lower or higher homologues, introduction or saturation of double bonds, introduction of optically active centers, introduction, removal or replacement of bulky groups, isosteric or bioisosteric substitution, changes in the position or orientation of a group, introduction of alkylating groups, and introduction, removal or replacement of groups with a view toward inhibiting or promoting inductive (electrostatic or conjugative (resonance) effects).

Thus, the substituents may include electron acceptors and/or electron donors. Typical electron donors (+I) include  $-\text{CH}_3$ ,  $-\text{CH}_2\text{R}$ ,  $-\text{CHR}_2$ ,  $-\text{CR}_3$  and  $-\text{COO}^-$ . Typical electron acceptors (-I) include  $-\text{NH}_3^+$ ,  $-\text{NR}_3^+$ ,  $-\text{NO}_2$ ,  $-\text{CN}$ ,  $-\text{COOH}$ ,  $-\text{COOR}$ ,  $-\text{CHO}$ ,  $-\text{COR}$ ,  $-\text{COR}$ ,  $-\text{F}$ ,  $-\text{Cl}$ ,  $-\text{Br}$ ,  $-\text{OH}$ ,  $-\text{OR}$ ,  $-\text{SH}$ ,  $-\text{SR}$ ,  $-\text{CH}=\text{CH}_2$ ,  $-\text{CR}=\text{CR}_2$ , and  $-\text{C}=\text{CH}$ .

The substituents may also include those which increase or decrease electronic density in conjugated systems. The former (+R) groups include  $-\text{CH}_3$ ,  $-\text{CR}_3$ ,  $-\text{F}$ ,  $-\text{Cl}$ ,  $-\text{Br}$ ,  $-\text{I}$ ,  $-\text{OH}$ ,  $-\text{OR}$ ,  $-\text{OCOR}$ ,  $-\text{SH}$ ,  $-\text{SR}$ ,  $-\text{NH}_2$ ,  $-\text{NR}_2$ , and  $-\text{NHCOR}$ . The later (-R) groups include  $-\text{NO}_2$ ,  $-\text{CN}$ ,  $-\text{CHC}$ ,  $-\text{COR}$ ,  $-\text{COOH}$ ,  $-\text{COOR}$ ,  $-\text{CONH}_2$ ,  $-\text{SO}_2\text{R}$  and  $-\text{CF}_3$ .

Synthetically speaking, the modifications may be achieved by a variety of unit processes, including nucleophilic and electrophilic substitution, reduction and oxidation, addition elimination, double bond cleavage, and cyclization.

For the purpose of constructing a library, a compound, or a family of compounds, having one or more pharmacological activities (which need not be related to the known or suspected activities of the target protein), may be disjoined into two or more known or potential pharmacophoric moieties. Analogues of each of these moieties may be identified, and mixtures of these analogues reacted so as to reassemble compounds which have some similarity to the original lead compound. It is not necessary that all members of the

library possess moieties analogous to all of the moieties of the lead compound.

The design of a library may be illustrated by the example of the benzodiazepines. Several benzodiazepine drugs, including chlordiazepoxide, diazepam and oxazepam, have been used on anti-anxiety drugs. Derivatives of benzodiazepines have widespread biological activities; derivatives have been reported to act not only as anxiolytics, but also as anticonvulsants, cholecystokinin (CCK) receptor subtype A or B, kappa opioid receptor, platelet activating factor, and HIV transactivator Tat antagonists, and GPIIbIIIa, reverse transcriptase and ras farnesyltransferase inhibitors.

The benzodiazepine structure has been disjoined into a 2-aminobenzophenone, an amino acid, and an alkylating agent. See Bunin, et al., Proc. Nat. Acad. Sci. USA, 91:4708 (1994). Since only a few 2-aminobenzophenone derivatives are commercially available, it was later disjoined into 2-aminoarylstannane, an acid chloride, an amino acid, and an alkylating agent. Bunin, et al., Meth. Enzymol., 267:448 (1996). The arylstannane may be considered the core structure upon which the other moieties are substituted, or all four may be considered equals which are conjoined to make each library member.

A basic library synthesis plan and member structure is shown in Figure 1 of Fowlkes, et al., U.S. Serial No. 08/740,671, incorporated by reference in its entirety. The acid chloride building block introduces variability at the R<sup>1</sup> site. The R<sup>2</sup> site is introduced by the amino acid, and the R<sup>3</sup> site by the alkylating agent. The R<sup>4</sup> site is inherent in the arylstannane. Bunin, et al. generated a 1, 4-benzodiazepine library of 11,200 different derivatives prepared from 20 acid chlorides, 35 amino acids, and 16 alkylating agents. (No diversity was introduced at R<sup>4</sup>; this group was used to couple the molecule to a solid phase.) According to the Available Chemicals Directory (HDL Information Systems, San Leandro CA), over 300 acid chlorides, 80 Fmoc-protected amino acids and 800 alkylating agents were available for purchase (and more, of course, could be synthesized). The particular moieties used

were chosen to maximize structural dispersion, while limiting the numbers to those conveniently synthesized in the wells of a microtiter plate. In choosing between structurally similar compounds, preference was given to the least substituted compound.

The variable elements included both aliphatic and aromatic groups. Among the aliphatic groups, both acyclic and cyclic (mono- or poly-) structures, substituted or not, were tested. (While all of the acyclic groups were linear, it would have been feasible to introduce a branched aliphatic). The aromatic groups featured either single and multiple rings, fused or not, substituted or not, and with heteroatoms or not. The secondary substituents included -NH<sub>2</sub>, -OH, -OMe, -CN, -Cl, -F, and -COOH. While not used, spacer moieties, such as -O-, -S-, -OO-, -CS-, -NH-, and -NR-, could have been incorporated.

Bunin et al. suggest that instead of using a 1, 4-benzodiazepine as a core structure, one may instead use a 1, 4-benzodiazepine-2, 5-dione structure.

As noted by Bunin et al., it is advantageous, although not necessary, to use a linkage strategy which leaves no trace of the linking functionality, as this permits construction of a more diverse library.

Other combinatorial nonoligomeric compound libraries known or suggested in the art have been based on carbamates, mercaptoacylated pyrrolidines, phenolic agents, aminimides, N-acylamino ethers (made from amino alcohols, aromatic hydroxy acids, and carboxylic acids), N-alkylamino ethers (made from aromatic hydroxy acids, amino alcohols and aldehydes) 1, 4-piperazines, and 1, 4-piperazine-6-ones.

DeWitt, et al., Proc. Nat. Acad. Sci. (USA), 90:6909-13 (1993) describes the simultaneous but separate, synthesis of 40 discrete hydantoins and 40 discrete benzodiazepines. They carry out their synthesis on a solid support (inside a gas dispersion tube), in an array format, as opposed to other conventional simultaneous synthesis techniques (e.g., in a well, or on a pin). The hydantoins were synthesized by first simultaneously deprotecting and then treating each of five amino acid resins with each of eight isocyanates. The

benzodiazepines were synthesized by treating each of five deprotected amino acid resins with each of eight 2-amino benzophenone imines.

Chen, et al., J. Am. Chem. Soc., 116:2661-62 (1994) described the preparation of a pilot (9 member) combinatorial library of formate esters. A polymer bead-bound aldehyde preparation was "split" into three aliquots, each reacted with one of three different ylide reagents. The reaction products were combined, and then divided into three new aliquots, each of which was reacted with a different Michael donor. Compound identity was found to be determinable on a single bead basis by gas chromatography/mass spectroscopy analysis.

Holmes, USP 5,549,974 (1996) sets forth methodologies for the combinatorial synthesis of libraries of thiazolidinones and metathiazanones. These libraries are made by combination of amines, carbonyl compounds, and thiols under cyclization conditions.

Ellman, USP 5,545,568 (1996) describes combinatorial synthesis of benzodiazepines, prostaglandins, beta-turn mimetics, and glycerol-based compounds. See also Ellman, USP 5,288,514.

Summerton, USP 5,506,337 (1996) discloses methods of preparing a combinatorial library formed predominantly of morpholino subunit structures.

Heterocyclic combinatorial libraries are reviewed generally in Nefzi, et al., Chem. Rev., 97:449-472 (1997). One or more moieties of the following types may be incorporated into compounds of the library, as many drugs fall into one or more of the following categories:

- 30        acetals
- acids
- alcohols
- amides
- amidines
- 35        amines
- amino acids



amino alcohols  
amino ethers  
amino ketenes  
ammonium compounds  
5 azo compounds  
enols  
esters  
ethers  
glycosides  
10 guanidines  
halogenated compounds  
hydrocarbons  
ketones  
lactams  
15 lactones  
mustards  
nitro compounds  
nitroso compounds  
organo minerals  
20 phenones  
quinones  
semicarbazones  
stilbenes  
sulfonamides  
25 sulfones  
thiols  
thioamides  
thioureas

ureas

ureides

urethans

Without attempting to exhaustively recite all  
5 pharmacological classes of drugs, or all drug structures, one  
or more compounds of the chemical structures listed below have  
been found to exhibit the indicated pharmacological activity,  
and these structures, or derivatives, may be used as design  
elements in screening for further compounds of the same or  
10 different activity. (In some cases, one or more lead drugs of  
the class are indicated.)

hypnotics

higher alcohols (clomethiazole)  
aldehydes (chloral hydrate)  
15 carbamates (meprobamate)  
acyclic ureides (acetylcarbromal)  
barbiturates (barbital)  
benzodiazepine (diazepam)

anticonvulsants

20 barbiturates (phenobarbital)  
hydantoins (phenytoin)  
oxazolidinediones (trimethadione)  
succinimides (phensuximide)  
acylureides (phenacemides)

25 narcotic analgesics

morphines  
phenylpiperidines (meperidine)  
diphenylpropylamines (methadone)  
phenothiazines (methotrimeprazine)

30 analgesics, antipyretics, antirheumatics

salicylates (acetylsalicylic acid)  
p-aminophenol (acetaminophen)  
5-pyrazolone (dipyrone)  
3, 5-pyrazolidinedione (phenylbutazone)

- arylacetic acid (indomethacin)
- adrenocortical steroids (cortisone, dexamethasone, prednisone, triamcilonone)
- athranilic acids
- 5        neuroleptics
  - phenothiazine (chlorpromazine)
  - thioxanthene (chlorprothixene)
  - reserpine
  - butyrophenone (halopendol)
- 10        anxiolytics
  - propandiol carbamates (meprobamate)
  - benzodiazepines        (chlordiazepoxide,        diazepam, oxazepam)
- 15        antidipressants
  - tricyclics (imipramine)
- muscle/relaxants
  - propanediols and carbamates (mephenesin)
- CNS stimulants
  - xanthines (caffeine, theophylline)
  - 20        phenylalkylamines (amphetamine)
    - (Fenetylline is a conjunction of theophylline and amphetamine)
  - oxazolidinones (pemoline)
- cholinergics
  - 25        choline esters (acetylcholine)
  - N,N-dimethylcarbamates
- adrenergics
  - aromatic amines (epinephrine, isoproterenol, phenylephrine)
  - 30        alicyclic amines (cyclopentamine)
  - aliphatic amines (methylhexaneamine)
  - imidazolines (naphazoline)

## anti-adrenergics

- indolethylamine alkaloids (dihydroergotamine)
- imidazoles (tolazoline)
- benzodioxans (piperoxan)
- 5 beta-haloalkylamines (phenoxybenzamine)
- dibenzazepines (azapetine)
- hydrazinophthalazines (hydralazine)

## antihistamines

- ethanolamines (diphenhydramine)
- 10 ethylenediamines (tripelennomine)
- alkylamines (chlorpheniramine)
- piperazines (cyclizine)
- phenothiazines (promethazine)

## local anesthetics

- 15 benzoic acid
- esters (procaine, isobucaine, cyclomethycaine)
- basic amides (dibucaine)
- anilides, toluidides, 2, 6-xylydides (lidocaine)
- tertiary amides (oxetacaine)

## 20 vasodilators

- polyol nitrates (nitroglycerin)

## diuretics

- xanthines
- thiazides (chlorothiazide)
- 25 sulfonamides (chlorthalidone)

## antihelmintics

- cyanine dyes

## antimalarials

- 4-aminoquinolines
- 30 8-aminoquinolines
- pyrimidines

- biguanides
- acridines
- dihydrotriazines
- sulfonamides
- 5 sulfones
- antibacterials
  - antibiotics
    - penicillins
    - cephalosporins
    - 10 octahydronaphthacenes (tetracycline)
    - sulfonamides
    - nitrofurans
    - cyclic amines
    - naphthyridines
    - 15 xylenols
- antitumor
  - alkylating agents
    - nitrogen mustards
    - aziridines
    - 20 methanesulfonate esters
    - epoxides
    - amino acid antagonists
    - folic acid antagonists
    - pyrimidine antagonists
    - 25 purine antagonists
- antiviral
  - adamantanes
  - nucleosides
  - thiosemicarbazones
  - 30 inosines
  - amidines and guanidines
  - isoquinolines
  - benzimidazoles
  - piperazines
  - 35 For pharmacological classes, see, e.g., Goth, Medical

- Pharmacology: Principles and Concepts (C.V. Mosby Co.: 8th ed. 1976); Korolkovas and Burckhalter, Essentials of Medicinal Chemistry (John Wiley & Sons, Inc.: 1976). For synthetic methods, see, e.g., Warren, Organic Synthesis: The  
5 Disconnection Approach (John Wiley & Sons, Ltd.: 1982); Fuson, Reactions of Organic Compounds (John Wiley & Sons: 1966); Payne and Payne, How to do an Organic Synthesis (Allyn and Bacon, Inc.: 1969); Greene, Protective Groups in Organic Synthesis (Wiley-Interscience). For selection of substituents, see e.g.,  
10 Hansch and Leo, Substituent Constants for Correlation Analysis in Chemistry and Biology (John Wiley & Sons: 1979).

The library is preferably synthesized so that the individual members remain identifiable so that, if a member is shown to be active, it is not necessary to analyze it. Several  
15 methods of identification have been proposed, including:

- (1) encoding, i.e., the attachment to each member of an identifier moiety which is more readily identified than the member proper. This has the disadvantage that the tag may itself influence the activity of  
20 the conjugate.
- (2) spatial addressing, e.g., each member is synthesized only at a particular coordinate on or in a matrix, or in a particular chamber. This might be, for example, the location of a particular pin, or a  
25 particular well on a microtiter plate, or inside a "tea bag".

The present invention is not limited to any particular form of identification.

However, it is possible to simply characterize those  
30 members of the library which are found to be active, based on the characteristic spectroscopic indicia of the various building blocks.

Solid phase synthesis permits greater control over which derivatives are formed. However, the solid phase could  
35 interfere with activity. To overcome this problem, some or all of the molecules of each member could be liberated, after synthesis but before screening.

Examples of candidate simple libraries which might be

evaluated include derivatives of the following:

Cyclic Compounds Containing One Hetero Atom

Heteronitrogen

- pyrroles
- 5            pentasubstituted pyrroles
- pyrrolidines
- pyrrolines
- prolines
- indoles
- 10          beta-carbolines
- pyridines
- dihydropyridines
- 1,4-dihydropyridines
- pyrido[2,3-d]pyrimidines
- 15          tetrahydro-3H-imidazo[4,5-c] pyridines
- Isoquinolines
- tetrahydroisoquinolines
- quinolones
- beta-lactams
- 20          azabicyclo[4.3.0]nonen-8-one amino acid
- Heterooxygen
- furans
- tetrahydrofurans
- 2,5-disubstituted tetrahydrofurans
- 25          pyrans
- hydroxypyranones
- tetrahydroxypyranones
- gamma-butyrolactones
- Heterosulfur
- 30          sulfolenes

Cyclic Compounds with Two or More Hetero atoms

Multiple heteronitrogens

- imidazoles
- pyrazoles
- 35          piperazines
- diketopiperazines
- arylpiperazines
- benzylpiperazines

- benzodiazepines
- 1,4-benzodiazepine-2,5-diones
- hydantoins
  - 5-alkoxyhydantoins
- 5 dihydropyrimidines
- 1,3-disubstituted-5,6-dihydropyrimidine-2,4-diones
- cyclic ureas
- cyclic thioureas
- 10 quinazolines
  - chiral3-substituted-quinazoline-2,4-diones
- triazotes
  - 1,2,3-triazoles
- purines
- 15 Heteronitrogen and Heterooxygen
  - dikelomorpholines
  - isoxazoles
  - isoxazolines
- Heteronitrogen and Heterosulfur
- 20 thiazolidines
  - N-axylthiazolidines
- dihydrothiazoles
  - 2-methylene-2,3-dihydrothiazates
  - 2-aminothiazoles
- 25 thiophenes
  - 3-amino thiophenes
- 4-thiazolidinones
- 4-melathiazanones
- benzisothiazolones
- 30 For details on synthesis of libraries, see Nefzi, et al.,  
Chem. Rev., 97:449-72 (1997), and references cited therein.



Amino Acids and Peptides

Amino acids are the basic building blocks with which peptides and proteins are constructed. Amino acids possess both an amino group ( $-NH_2$ ) and a carboxylic acid group ( $-COOH$ ).

5 Many amino acids, but not all, have the structure  $NH_2-CHR-COOH$ , where R is hydrogen, or any of a variety of functional groups.

Twenty amino acids are genetically encoded: Alanine, Arginine, Asparagine, Aspartic Acid, Cysteine, Glutamic Acid, Glutamine, Glycine, Histidine, Isoleucine, Leucine, Lysine,  
10 Methionine, Phenylalanine, Proline, Serine, Threonine, Tryptophan, Tyrosine, and Valine. Of these, all save Glycine are optically isomeric, however, only the L-form is found in humans. Nevertheless, the D-forms of these amino acids do have biological significance; D-Phe, for example, is a known  
15 analgesic.

Many other amino acids are also known, including: 2-Aminoadipic acid; 3-Aminoadipic acid; beta-Aminopropionic acid; 2-Aminobutyric acid; 4-Aminobutyric acid (Piperidinic acid); 6-Aminocaproic acid; 2-Aminoheptanoic acid; 2-Aminoisobutyric  
20 acid, 3-Aminoisobutyric acid; 2-Aminopimelic acid; 2,4-Diaminobutyric acid; Desmosine; 2,2'-Diaminopimelic acid; 2,3-Diaminopropionic acid; N-Ethylglycine; N-Ethylasparagine; Hydroxylysine; allo-Hydroxylysine; 3-Hydroxyproline; 4-Hydroxyproline; Isodesmosine; allo-Isoleucine; N-  
25 Methylglycine (Sarcosine); N-Methylisoleucine; N-Methylvaline; Norvaline; Norleucine; and Ornithine.

Peptides are constructed by condensation of amino acids and/or smaller peptides. The amino group of one amino acid (or peptide) reacts with the carboxylic acid group of a second  
30 amino acid (or peptide) to form a peptide ( $-NHCO-$ ) bond, releasing one molecule of water. Therefore, when an amino acid is incorporated into a peptide, it should, technically speaking, be referred to as an amino acid residue.

A peptide is composed of a plurality of amino acid  
35 residues joined together by peptidyl ( $-NHCO-$ ) bonds. A biogenic peptide is a peptide in which the residues are all genetically encoded amino acid residues; it is not necessary that the biogenic peptide actually be produced by gene

expression.

The peptides of the present invention include peptides whose sequences are disclosed in this specification, or sequences differing from the above solely by no more than one nonconservative substitution and/or one or more conservative substitutions, preferably no more than a single conservative substitution. The substitutions may be of non-genetically encoded (exotic) amino acids, in which case the resulting peptide is nonbiogenic.

A conservative substitution is a substitution of one amino acid for another of the same exchange group, the exchange groups being defined as follows

- I Gly, Pro, Ser, Ala (Cys) (and any nonbiogenic, neutral amino acid with a hydrophobicity not exceeding that of the aforementioned a.a.'s)
- II Arg, Lys, His (and any nonbiogenic, positively-charged amino acids)
- III Asp, Glu, Asn, Gln (and any nonbiogenic negatively-charged amino acids)
- IV Leu, Ile, Met, Val (Cys) (and any nonbiogenic, aliphatic, neutral amino acid with a hydrophobicity too high for I above)
- V Phe, Trp, Tyr (and any nonbiogenic, aromatic neutral amino acid with a hydrophobicity too high for I above).

Note that Cys belongs to both I and IV.

A highly conservative substitution, which is preferred, is Arg/Lys/His, Asp/Glu, Asn/Gln, Leu/Ile/Met/Val, Phe/Trp/Tyr, or Gly/Ser/Ala.

Additional peptides within the present invention may be identified by systematic mutagenesis of the lead peptides, e.g.

- (a) separate synthesis of all possible single substitution (especially of genetically encoded AAs) mutants of each lead peptide, and/or
- (b) simultaneous binomial random alanine-scanning mutagenesis of each lead peptide, so each amino acids position may be either the original amino acid or alanine (alanine being a semi-conservative

substitution for all other amino acids), and/or  
(c) simultaneous random mutagenesis sampling  
conservative substitutions of some or all positions  
of each lead peptide, the number of sequences in  
5 total sequences space for a given experiment being  
such that any sequence, if active, is within  
detection limits (typically, this means not more  
than about  $10^{10}$  different sequences).

The mutants are tested for activity, and, if active, are  
10 considered to be within "peptides of the present invention".  
Even inactive mutants contribute to our knowledge of structure-  
activity relationships and thus assist in the design of  
peptides, peptoids, and peptidomimetics.

Preferably, substitutions of exotic amino acids for the  
15 original amino acids take the form of

- (I) replacement of one or more hydrophilic amino  
acid side chains with another hydrophilic  
organic radical, not more than twice the volume  
of the original side chain, or
- 20 (II) replacement of one or more hydrophobic amino  
acid side chains with another hydrophobic  
organic radical, not more than twice the volume  
of the original side chain.

The exotic amino acids may be alpha or non-alpha amino  
25 acids (e.g., beta alanine). They may be alpha amino acids with  
2 R groups on the  $C\alpha$ , which groups may be the same or  
different. They may be dehydro amino acids ( $HOOC-C(NH_2)=CHR$ ).

For further information on synthesis of peptides including  
exotic amino acids, see:

- 30 1. Bielfeldt, T., Peters, S., Meldal, M., Bock, K. and  
Paulsen, N.A. new strategy for solid-phase synthesis of O-  
glycopeptides. *Angew. Chem. (Engl)* 31:857-859, 1992.
- 2. Gurjar, M.K. and Saha, U.K. Synthesis of the  
glycopeptide-O-(3,4-di-O-methyl-2-O-[3,4-di-O-methyl- $\alpha$ -L-  
35 rhamnopyranosyl]- $\alpha$ -L-rhamnopyranosyl)-L-alaninol: An unusual  
part structure in the glycopeptidolipid of Mycobacterium  
fortuitum. *Tetrahedron* 48:4039-4044, 1992.
- 3. Kessler, H., Wittmann, V., Kock, M. and Kottenhahn,

- M. Synthesis of C-glycopeptides via free radical addition of glycosyl bromides to dehydroalanine derivatives. *Angew. Chem. (Engl.)* 31:902-904, 1992.
4. Kraus, J.L. and Attardo, G. Synthesis and biological activities of new N-formylated methionyl peptides containing an  $\alpha$ -substituted glycine residue. *European Journal of Medicinal Chemistry* 27:19-26, 1992.
5. Mhaskar, S.Y. Synthesis of N-lauroyl dipeptides and correlation of their structure with surfactant and antibacterial properties. *J. Am. Oil Chem. Soc.* 69:647-652, 1992.
6. Moree, W.J., Van der Marel, G.A. and Liskamp, R.M.J. Synthesis of peptides containing the  $\beta$ -substituted aminoethane sulfinamide or sulfonamide transition-state isostere derived from amino acids. *Tetrahedron Lett.* 33:69-6392, 1992.
7. Paquet, A. Further studies on the use of 2,2,2-trichloroethyl groups for phosphate protection in phosphoserine peptide synthesis. *International Journal of Peptide and Protein Research* 39:82-86, 1992.
8. Sewald, N., Riede, J., Bissinger, P. and Burger, K. A new convenient synthesis of 2-trifluoromethyl substituted aspartic acid and its isopeptides. Part 11. *Journal of the Chemical Society. Perkin Transactions 1* 1992:267-274, 1992.
9. Simon, R.J., Kania, R.S., Zuckermann, R.N., Huebner, V.D., Jewell, D.A., Banville, S., Ng, S., Wang, L., Rosenberg, S., Marlowe, C.K., Spellmeyer, D.C., Tan, R., Frankel, A.D., Santi, D.V., Cohen, F.E. and Bartlett, P.A. Peptoids: A modular approach to drug discovery. *Proc. Natl. Acad. Sci. USA* 89:9367-9371, 1992.
10. Tung, C.-H., Zhu, T., Lackland, H. and Stein, S. An acridine amino acid derivative for use in Fmoc peptide synthesis. *Peptide Research* 5:115-118, 1992.
11. Elofsson, M. Building blocks for glycopeptide synthesis: Glycosylation of 3-mercaptopropionic acid and Fmoc amino acids with unprotected carboxyl groups. *Tetrahedron Lett.* 32:7613-7616, 1991.
12. McMurray, J.S. Solid phase synthesis of a cyclic peptide using Fmoc chemistry. *Tetrahedron Letters* 32:7679-

7682, 1991.

13. Nunami, K.-I., Yamazaki, T. and Goodman, M. Cyclic retro-inverso dipeptides with two aromatic side chains. I. Synthesis. *Biopolymers* 31:1503-1512, 1991.
- 5 14. Rovero, P, Synthesis of cyclic peptides on solid support. *Tetrahedron Letters* 32:2639-2642, 1991.
- 15 15. Elofsson, M., Walse, B. and Kihlberg, J. Building blocks for glycopeptide synthesis: Glycosylation of 3-mercaptopropionic acid and Fmoc amino acids with unprotected  
10 carboxyl groups. *Tetrahedron Letter*, 32:7613-7616, 1991.
16. Bielfeldt, T., Peter, S., Meldal, M., Bock, K. and Paulsen, H. A new strategy for solid-phase synthesis of O-glycopeptides. *Angew. Chem (Engl)* 31:857-859, 1992.
- 15 17. Luning, B., Norberg, T. and Tejbrant, J. Synthesis of glycosylated amino acids for use in solid phase glycopeptide synthesis, par 2:N-(9-fluorenylmethyloxycarbonyl)-3-O-[2,4,6-tri-O-acetyl- $\alpha$ -D-sylopyranosyl]- $\beta$ -D-glucopyranosyl]-L-serine. *J. Carbohydr. Chem.* 11:933-943, 1992.
- 20 18. Peters, S., Bielfeldt, T., Meldal, M., Bock, K. and Paulsen, H. Solid phase peptide synthesis of mucin glycopeptides. *Tetrahedron Lett.* 33:6445-6448, 1992.
- 25 19. Urge, L., Otvos, L., Jr., Lang, E., Wroblewski, K., Laczko, I. and Hollosi, M. Fmoc-protected, glycosylated asparagines potentially useful as reagents in the solid-phase synthesis of N-glycopeptides, *Carbohydr. Res.* 235:83-93, 1992.
20. Gerz, M., Matter, H. and Kessler, H., S-glycosylated cyclic peptides, *Angew. Chem. (Engl.)* 32:269-271, 1993.

### Cyclic Peptides

Many naturally occurring peptide are cyclic. Cyclization  
30 is a common mechanism for stabilization of peptide conformation thereby achieving improved association of the peptide with its ligand and hence improved biological activity. Cyclization is usually achieved by intra-chain cystine formation, by formation of peptide bond between side chains or between N- and C-  
35 terminals. Cyclization was usually achieved by peptides in solution, but several publications have appeared recently that describe cyclization of peptides on beads (see references

below).

1. Spatola, A.F., Anwer, M.K. and Rao, M.N. Phase transfer catalysis in solid phase peptide synthesis. Preparation of cycle [Xxx-Pro-Gly-Yyy-Pro-Gly] model peptides and their conformational analysis. *Int. J. Pept. Protein Res.* 40:322-332, 1992.
2. Tromelin, A., Fulachier, M.-H., Mourier, G. and Menez, A. Solid phase synthesis of a cyclic peptide derived from a curaremimetic toxin. *Tetrahedron Lett.* 33:5197-5200, 1992.
3. Trzeciak, A. Synthesis of 'head-to-tail' cyclized peptides on solid supports by Fmoc chemistry. *Tetrahedron Lett.* 33:4557-45560, 1992.
4. Wood, S. J. and Wetzel, R. Novel cyclization chemistry especially suited for biologically derived, unprotected peptides, *Int. J. Pept. Protein Res.* 39:533-539, 1992.
5. Gilon, C., Halle, D., Chorev, M., Selinger, Z. and Byk, G. Backbone cyclization: A new method for conferring conformational constraint on peptides. *Biopolymers* 31:745-750, 1991.
6. McMurray, J. S. Solid phase synthesis of a cyclic peptide using Fmoc chemistry. *Tetrahedron Letters* 32:7679-7682, 1991.
7. Rovero, P. Synthesis of cyclic peptides on solid support. *Tetrahedron Letters* 32:2639-2642, 1991.
8. Yajima, X. Cyclization on the bead via following Cys Acn deprotection. *Tetrahedron* 44:805, 1988.

### Peptoid

- 30 A peptoid is an analogue of a peptide in which one or more of the peptide bonds are replaced by pseudopeptide bonds, which may be the same or different.

Such pseudopeptide bonds may be:

- Carba  $\Psi(\text{CH}_2-\text{CH}_2)$   
35 Depsi  $\Psi(\text{CO}-\text{O})$   
Hydroxyethylene  $\Psi(\text{CHOH}-\text{CH}_2)$   
Ketomethylene  $\Psi(\text{CO}-\text{CH}_2)$

Methylene-ocy  $\text{CH}_2\text{-O-}$

Reduced  $\text{CH}_2\text{-NH}$

Thiomethylene  $\text{CH}_2\text{-S-}$

Thiopeptide  $\text{CS-NH}$

5 N-modified -NRCO-

See also

1. Corringier, P.J., Weng, J.H., Ducos, B., Durieux, C., Boudeau, P., Bohme, A. and Roques, B.P. CCK-B agonist or antagonist activities of structurally hindered and peptidase-resistant Boc-CCK<sub>4</sub> derivatives. *J. Med. Chem.* 36:166-172, 1993. Amino acids reported: aromatic naphthylalaninimide (Nal-NH<sub>2</sub>); N-methyl amino acids.
2. Beylin, V.G., Chen, H.G., Dunbar, J., Goel, O.P., Harter, W., Marlatt, M. and Topliss, J.G. Cyclic derivatives of 3,3-diphenylalanine (Dip) (II), novel  $\alpha$ -amino acids for peptides of biological interest. *Tetrahedron Lett.* 34:953-956, 1993.
3. Garbay-Jaureguiberry, C., Ficheux, D. and Roques, B.P. Solid phase synthesis of peptides containing the non-glycolysable analog of (O)phosphotyrosine,  $p(\text{CH}_2\text{PO}_3\text{H}_2)\text{Phe}$ . Application to the synthesis of 344-357 sequences of the  $\beta_2$  adrenergic receptor. *Int. J. Pept. Protein Res.* 39:523-527, 1992.
4. Lüning, B., Norberg, T. and Tejbrant, J. Synthesis of glycosylated amino acids for use in solid phase glycopeptide synthesis, part 2: N-(9-fluorenylmethyloxycarbonyl)-3-O-[2,4,6-tri-O-acetyl-3-O-(2,3,4-tri-O-acetyl- $\alpha$ -D-xylopyranosyl)- $\beta$ -D-glucopyranosyl]-L]serine. *J. Carbohydr. Chem.* 11:933-943, 1992.
5. Tung, C.H., Zhu, T., Lackland, H. and Stein, S. An acridine amino acid derivative for use in Fmoc peptide synthesis. *Peptide Research* 5:115-118, 1992.
6. Eric Frerot, PyBOP and PyBroP: Two reagents for the difficult coupling of the  $\alpha,\alpha$ -dialkyl amino acid Aib. *Tetrahedron* 47:259-270, 1991.
7. Moree, W.J., Van der Marel, G.A. and Liskamp, R.M.J. Synthesis of peptides containing the  $\beta$ -substituted aminoethane sulfinamide or sulfonamide transition-state isostere derived

- from amino acids. *Tetrahedron Lett.* 33:6389-6392, 1992.
8. Rana, T.M. Synthesis of a metal-binding amino acid suitable for solid phase assembly of peptides. *Tetrahedron Lett.* 33:4521-4524, 1992.
- 5 9. Urge, L., Otvos, L., Jr., Lang, E., Wroblewski, K., Laczko, I. and Hollosi, M. Fmoc-protected, glycosylated asparagines potentially useful as reagents in the solid-phase synthesis of N-glycopeptides. *Carbohydr. Res.* 235:83-93, 1992.
- 10 10. Pavone, V., DiBlasio, B., Lombardi, A., Maglio, O., Isernia, D., Pedone, C., Benedette, E., Altmann, E. and Mutter, M. Non coded C $\alpha$ -disubstituted amino acids. X-ray diffraction analysis of a dipeptide containing (S)- $\alpha$ -methylserine. *Int. J. Pept. Protein Res.* 41:15-20, 1993.
- 15 11. Nishino, N., Mihara, H., Kiyota, H., Kobata, K. and Fujimoto, T. Aminoporphyrinic acid as a new template for polypeptide design. *J. Chem. Soc. Chem. Commun.* 1993:162-163, 1993.
- 20 12. Sosnovsky, G., Prakash, I. and Rao, N.U.M. In the search for new anticancer drugs. XXIV: Synthesis and anticancer activity of amino acids and dipeptides containing the 2-chloroethyl- and [N'-nitroso]-aminocarbonyl groups. *J. Pharm. Sci.* 82:1-10, 1993.
- 25 13. Berti, F., Ebert, C. and Gardossi, L. One-step stereospecific synthesis of  $\alpha,\beta$ -dehydroamino acids and dehydropeptides. *Tetrahedron Lett.* 33:8145-8148, 1992.

### Peptidomimetic

A peptidomimetic is a molecule which mimics the biological activity of a peptide, by substantially duplicating the pharmacologically relevant portion of the conformation of the peptide, but is not a peptide or peptoid as defined above. Preferably the peptidomimetic has a molecular weight of less than 700 daltons.

Designing a peptidomimetic usually proceeds by:

- 35 (a) identifying the pharmacophoric groups responsible for the activity;
- (b) determining the spatial arrangements of the pharmacophoric groups in the active conformation of



the peptide; and

- (c) selecting a pharmaceutically acceptable template upon which to mount the pharmacophoric groups in a manner which allows them to retain their spatial arrangement in the active conformation of the peptide.

Step (a) may be carried out by preparing mutants of the active peptide and determining the effect of the mutation on activity. One may also examine the 3D structure of a complex of the peptide and the receptor for evidence of interactions, e.g., the fit of a side chain of the peptide into a cleft of the receptor; potential sites for hydrogen bonding, etc.).

Step (b) generally involves determining the 3D structure of the active peptide, in the complex, by NMR spectroscopy or X-ray diffraction studies. The initial 3D model may be refined by an energy minimization and molecular dynamics simulation.

Step (c) may be carried out by reference to a template database, see Wilson, et al. Tetrahedron, 49:3655-63 (1993). The templates will typically allow the mounting of 2-8 pharmacophores, and have a relatively rigid structure. For the latter reason, aromatic structures, such as benzene, biphenyl, phenanthrene and benzodiazepine, are preferred. For orthogonal protection techniques, see Tuchscherer, et al., Tetrahedron, 17:3559-75 (1993).

For more information on peptoids and peptidomimetics, see USP 5,811,392, USP 5,811,512, USP 5,578,629, USP 5,817,879, USP 5,817,757, USP 5,811,515.

#### Analogues

Also of interest are analogues of the disclosed peptides, and other compounds with activity of interest.

Analogues may be identified by assigning a hashed bitmap structural fingerprint to the compound, based on its chemical structure, and determining the similarity of that fingerprint to that of each compound in a broad chemical database. The fingerprints are determined by the fingerprinting software commercially distributed for that purpose by Daylight Chemical Information Systems, Inc., according to the software release

current as of January 8, 1999. In essence, this algorithm generates a bit pattern for each atom, and for its nearest neighbors, with paths up to 7 bonds long. Each pattern serves as a seed to a pseudorandom number generator, the output of which is a set of bits which is logically ored to the developing fingerprint. The fingerprint may be fixed or variable size.

The database may be SPRESI'95 (InfoChem GmbH), Index Chemicus (ISI), MedChem (Pomona/Biobyte), World Drug Index (Derwent), TSCA93(EPA) May bridge organic chemical catalog (Maybridge), Available Chemicals Directory (MDLIS Inc.), NCI96 (NCI), Asinex catalog of organic compounds (Asinex Ltd.), or IBIOScreen SC and NP (Inter BioScreen Ltd.), or an inhouse database.

A compound is an analogue of a reference compound if it has a daylight fingerprint with a similarity (Tanamoto coefficient) of at least 0.85 to the Daylight fingerprint of the reference compound.

A compound is also an analogue of a reference compound if it may be conceptually derived from the reference compound by isosteric replacements.

Homologues are compounds which differ by an increase or decrease in the number of methylene groups in an alkyl moiety.

Classical isosteres are those which meet Erlenmeyer's definition: "atoms, ions or molecules in which the peripheral layers of electrons can be considered to be identical". Classical isosteres include

<u>Monovalents</u>	<u>Bivalents</u>	<u>Trivalents</u>	<u>Tetra</u>	<u>Annular</u>
F, OH, NH <sub>2</sub> , CH <sub>3</sub>	-O-	-N=	=C=	-CH=CH-
			=Si=	
Cl, SH, PH <sub>2</sub>	-S-	-P=	-N+=	-S-
Br	-Se-	-As-	=P+=	-O-
i	-Te-	-Sb-	=As+=	-NH-
		-CH=	=Sb+=	

Nonclassical isosteric pairs include -CO- and -SO<sub>2</sub>-, -COOH and -SO<sub>3</sub>H, -SO<sub>2</sub>NH<sub>2</sub> and -PO(OH)NH<sub>2</sub>, and -H and -F, -OC(=O)- and C(=O)O-, -OH and -NH<sub>2</sub>.

Pharmaceutical Methods and Preparations

The preferred animal subject of the present invention is a mammal. By the term "mammal" is meant an individual belonging to the class Mammalia. The invention is particularly  
5 useful in the treatment of human subjects, although it is intended for veterinary uses as well. Preferred nonhuman subjects are of the orders Primata (e.g., apes and monkeys), Artiodactyla or Perissodactyla (e.g., cows, pigs, sheep, horses, goats), Carnivora (e.g., cats, dogs), Rodenta (e.g.,  
10 rats, mice, guinea pigs, hamsters), Lagomorpha (e.g., rabbits) or other pet, farm or laboratory mammals.

The term "protection", as used herein, is intended to include "prevention," "suppression" and "treatment." "Prevention" involves administration of the protein prior to  
15 the induction of the disease (or other adverse clinical condition). "Suppression" involves administration of the composition prior to the clinical appearance of the disease. "Treatment" involves administration of the protective composition after the appearance of the disease.

20 It will be understood that in human and veterinary medicine, it is not always possible to distinguish between "preventing" and "suppressing" since the ultimate inductive event or events may be unknown, latent, or the patient is not ascertained until well after the occurrence of the event or  
25 events. Therefore, it is common to use the term "prophylaxis" as distinct from "treatment" to encompass both "preventing" and "suppressing" as defined herein. The term "protection," as used herein, is meant to include "prophylaxis." It should also be understood that to be useful, the protection provided need  
30 not be absolute, provided that it is sufficient to carry clinical value. An agent which provides protection to a lesser degree than do competitive agents may still be of value if the other agents are ineffective for a particular individual, if it can be used in combination with other agents to enhance the  
35 level of protection, or if it is safer than competitive agents. The drug may provide a curative effect, an ameliorative effect, or both.

At least one of the drugs of the present invention may be

administered, by any means that achieve their intended purpose, to protect a subject against a disease or other adverse condition. The form of administration may be systemic or topical. For example, administration of such a composition may  
5 be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. Parenteral administration can be by bolus injection or by  
10 gradual perfusion over time.

A typical regimen comprises administration of an effective amount of the drug, administered over a period ranging from a single dose, to dosing over a period of hours, days, weeks, months, or years.

15 It is understood that the suitable dosage of a drug of the present invention will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. However, the most preferred dosage can be tailored  
20 to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. This will typically involve adjustment of a standard dose, e.g., reduction of the dose if the patient has a low body weight.

25 Prior to use in humans, a drug will first be evaluated for safety and efficacy in laboratory animals. In human clinical studies, one would begin with a dose expected to be safe in humans, based on the preclinical data for the drug in question, and on customary doses for analogous drugs (if any). If this  
30 dose is effective, the dosage may be decreased, to determine the minimum effective dose, if desired. If this dose is ineffective, it will be cautiously increased, with the patients monitored for signs of side effects. See, e.g., Berkow et al, eds., *The Merck Manual*, 15th edition, Merck and Co., Rahway,  
35 N.J., 1987; Goodman et al., eds., *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 8th edition, Pergamon Press, Inc., Elmsford, N.Y., (1990); Avery's *Drug Treatment: Principles and Practice of Clinical Pharmacology and*

*Therapeutics*, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, MD. (1987), Ebadi, *Pharmacology*, Little, Brown and Co., Boston, (1985), which references and references cited therein, are entirely incorporated herein by reference.

5       The total dose required for each treatment may be administered by multiple doses or in a single dose. The protein may be administered alone or in conjunction with other therapeutics directed to the disease or directed to other symptoms thereof.

10       The appropriate dosage form will depend on the disease, the protein, and the mode of administration; possibilities include tablets, capsules, lozenges, dental pastes, suppositories, inhalants, solutions, ointments and parenteral depots. See, e.g., Berker, *supra*, Goodman, *supra*, Avery, *supra*  
15 and Ebadi, *supra*, which are entirely incorporated herein by reference, including all references cited therein.

      In the case of peptide drugs, the drug may be administered in the form of an expression vector comprising a nucleic acid encoding the peptide, such a vector, after incorporation into  
20 the genetic complement of a cell of the patient, directs synthesis of the peptide. Suitable vectors include genetically engineered poxviruses (vaccinia), adenoviruses, adeno-associated viruses, herpesviruses and lentiviruses which are or have been rendered nonpathogenic.

25       In addition to at least one drug as described herein, a pharmaceutical composition may contain suitable pharmaceutically acceptable carriers, such as excipients, carriers and/or auxiliaries which facilitate processing of the active compounds into preparations which can be used  
30 pharmaceutically. See, e.g., Berker, *supra*, Goodman, *supra*, Avery, *supra* and Ebadi, *supra*, which are entirely incorporated herein by reference, included all references cited therein.

#### Anti-Cancer Utility

      One utility of certain ER-binding peptides of the present  
35 invention, and related peptoids, peptidomimetics and analogues, and compounds fingerprinted as sensitive to the interaction of such peptides with ER, is in circumventing tamoxifen resistance

in breast cancer.

It is now estimated that the lifetime risk among American women of being diagnosed with breast cancer is about one in eight. Although this figure represents a doubling of the incidence of this disease over the past fifty years, it is counterbalanced by the observation that mortality from this disease has decreased slightly over the same period. In the recent NSAPB-B14 trial it was demonstrated that the 10 year survival rate in breast cancer patients who were node negative at time of diagnosis was greater than 80%. It is likely that this favorable response is due in large part to advances in early detection which has had the effect of decreasing the number of women who present with metastatic disease to more manageable early stage malignancies. In addition to early detection however, the strategic use of the antiestrogen tamoxifen for the treatment of metastatic disease and as an adjuvant chemotherapeutic has had a positive impact on survival in breast cancer patients. One of the most dramatic benefits of tamoxifen is that it reduces the incidence of contralateral primary tumors in patients by greater than 50%. It was this finding, combined with the results of the NSABP-P1 chemoprevention trial, which led recently to the approval of tamoxifen for use as a breast cancer chemopreventative in women who are at an elevated risk for breast cancer. Clearly, tamoxifen is an extremely successful pharmaceutical. As with most drugs however, the effectiveness of tamoxifen as a chemotherapeutic agent decreases with time. In the metastatic setting, it has been observed that most tamoxifen responsive breast cancers eventually become resistant to its antiestrogenic actions. A decrease in effectiveness over time in the adjuvant setting is also inferred from the results of the NSABP-B14 trial which demonstrated that the overall survival rate of breast cancer patients who were asking tamoxifen for 10 years was no better, and possibly even worse, than women who took this drug for only five years. The latter result has led to the suggestion that the tumors in patients who were on tamoxifen for extended periods of time may lose the ability to recognize this drug as an antiestrogen and may

in fact change in some manner to respond to the drug as an estrogen. The observation that some patients display a withdrawal response when tamoxifen administration is discontinued supports this hypothesis. Consequently, there has  
5 been a tremendous amount of interest in understanding the process by which breast tumors fail tamoxifen and in the application of this knowledge to the development of novel antiestrogens with improved therapeutic benefits.

Several years ago it was considered unlikely that the  
10 estrogen receptor (ER) would be a useful target in those cells which have failed tamoxifen. However, the emergence of pure antiestrogens, like ICI182,780, which have been used successfully to treat tamoxifen refractory breast cancers has validated ER as a target in this stage of the disease.  
15 However, since they non-selectively block estrogen action in all target organs they will have a negative impact in the skeletal and cardiovascular systems and consequently will not be suitable for use as adjuvant chemotherapeutics. There is an unmet medical need therefore, for novel antiestrogens which  
20 are mechanistically distinct from tamoxifen in the breast but which retain the positive estrogenic actions of tamoxifen in the bone and the cardiovascular systems.

Tamoxifen was developed originally as an antiestrogen which could be used to block the actions of estrogen at the  
25 receptor level in breast cancer cells. Thus, it was generally held that resistance to this agent occurred as a consequence of ER mutations, selective extrusion of the compound from cells or as a result of inactivating metabolic processes. However, it now appears that these mechanisms only explain tamoxifen  
30 resistance in a small percentage of cases. Other mechanisms are now being considered. We favor a model in which epigenetic changes occur within target cells affecting their ability to recognize tamoxifen as an antagonist and may in fact permit them to recognize the drug as an estrogenic ligand. This  
35 hypothesis stems from the observation that tamoxifen is in fact a selective estrogen receptor modulator (SERM) which can function as an ER antagonist, or an agonist, depending on the cell background in which it is studied. Thus, we believe that

in breast the selective pressure of tamoxifen promotes the outgrowth of a population of cells, through accommodation or selection, which recognize tamoxifen as an agonist. Consequently, we and others, have focused on defining the molecular basis for the cell selective actions of tamoxifen, and other SERMs, with a view to understanding tamoxifen resistance and the eventual development of novel antiestrogens. These studies have revealed that upon binding ligand, ER undergoes a conformational change, the nature of which is influenced by the structure of the bound ligand. The significance of these conformational changes was revealed when it was determined that ER contains two activation domains, AF-1 located at the amino terminus and AF-2 contained within the hormone binding domain, the activity of which is influenced by both cell and promoter context. In most cells both AFs are required for maximal transcriptional activity. Accordingly, it has been shown that estradiol functions as an ER agonist in all cells as it facilitates the interaction of both AFs with the transcription apparatus. It has now been determined that tamoxifen alters ER structure in a manner which inhibits AF-2 function. Thus, in all contexts where AF-2 is required, tamoxifen manifests antagonist activity. In cell contexts where AF-1 alone is sufficient for ER transcriptional activity we have determined that tamoxifen can function as a partial agonist. This finding led us to hypothesize that the residual agonist activity of tamoxifen, observed in AF-1 dominant environments, may be linked to the failure of this drug as an antiestrogen in breast cancer. Thus, we searched for compounds which did not activate AF-1 and evaluated their ability to inhibit tamoxifen partial agonist activity. This work led to the discovery of a novel antiestrogen, GW5638, which when assayed in vitro, inhibits tamoxifen partial agonist activity under all conditions examined and effectively inhibited the growth of MCF-7 cell xenografts in A-thymic nude mice. Because of these properties, GW5638 will soon enter clinical trails for evaluation as a treatment of tamoxifen refractory breast cancer.

One of the surprising properties of the novel



antiestrogen, GW5638, is that although it is devoid of AF-1 and AF-2 agonist activity it is not a pure antagonist when assayed in vivo. Unlike tamoxifen, it does not display uterotrophic activity. However, like tamoxifen, it functions as an estrogen in bone and the cardiovascular system. These results indicate that the ability to differentially activate AF-1 and AF-2 may be important but that the pharmacology of this class of antiestrogens is more complex than we anticipated. Consequently, we have focused recently on defining the molecular mechanism(s) by which cells distinguish between tamoxifen and GW5638. Although still ongoing, it has led to the development of a novel approach to inhibit the partial agonist activity of tamoxifen. Specifically, using phage display technology we have identified small peptides whose interaction with ER is influenced by the nature of the bound ligand. Peptides have been found which interact with ER in the presence of any ligand, in the presence of any agonist, in the presence of any antagonist and more importantly, we have identified peptides which interact with ER only in the presence of tamoxifen. With respect to the development of strategies to treat tamoxifen refractory breast cancer, the latter peptides are the most interesting as we have shown in vitro that these peptides efficiently inhibit tamoxifen partial agonist activity. Mapping of the sites on ER with which these peptides interact will help in determining if they mimic specific coactivator interactions. Regardless however, this work has defined several sites on ER that will serve as targets for new drug discovery. Although peptides do not generally serve as good starting places for drug development, there has been a tremendous amount of progress of late in generating small molecules which modulate protein-protein interactions. Consequently, we are now in the process of screening for small molecules which interact with the target sites implicated by the novel peptides and additionally are in the process of defining smaller peptides which in themselves may be useful, if suitably formulated, as drugs.

#### Binding Molecule

For the purpose of the discussion of diagnostic methods and agents which follows, the "binding molecule" is the peptide, peptoid or peptidomimetic of the present invention. The analyte is a target protein.

5 In Vitro Diagnostic Methods and Reagents

The in vitro assays of the present invention may be applied to any suitable analyte-containing sample, and may be qualitative or quantitative in nature. In order to detect the presence, or measure the amount, of an analyte, the assay must  
10 provide for a signal producing system (SPS) in which there is a detectable difference in the signal produced, depending on whether the analyte is present or absent (or, in a quantitative assay, on the amount of the analyte). The detectable signal may be one which is visually detectable, or one detectable only  
15 with instruments. Possible signals include production of colored or luminescent products, alteration of the characteristics (including amplitude or polarization) of absorption or emission of radiation by an assay component or product, and precipitation or agglutination of a component or  
20 product. The term "signal" is intended to include the discontinuance of an existing signal, or a change in the rate of change of an observable parameter, rather than a change in its absolute value. The signal may be monitored manually or automatically.

25 The component of the signal producing system which is most intimately associated with the diagnostic reagent is called the "label". A label may be, e.g., a radioisotope, a fluorophore, an enzyme, a co-enzyme, an enzyme substrate, an electron-dense compound, an agglutinable particle. One diagnostic reagent is  
30 a conjugate, direct or indirect, or covalent or noncovalent, of a label with a binding molecule of the invention.

The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for  
35 the purpose of the present invention are  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ , and, preferably,  $^{125}\text{I}$ .

It is also possible to label a compound with a fluorescent

compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

Alternatively, fluorescence-emitting metals such as  $^{125}\text{Eu}$ , or others of the lanthanide series, may be attached to the binding protein using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediamine-tetraacetic acid (EDTA).

The binding molecules also can be detectably labeled by coupling to a chemiluminescent compound. The presence of the chemiluminescent compound is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction after a suitable reactant is provided. Examples of particularly useful chemiluminescent labeling compounds are luminol, isolumino, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the binding molecule. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Enzyme labels, such as horseradish peroxidase and alkaline phosphatase, are preferred. When an enzyme label is used, the signal producing system must also include a substrate for the enzyme. If the enzymatic reaction product is not itself detectable, the SPS will include one or more additional reactants so that a detectable product appears.

Assays may be divided into two basic types, heterogeneous and homogeneous. In heterogeneous assays, the interaction between the affinity molecule and the analyte does not affect the label, hence, to determine the amount or presence of analyte, bound label must be separated from free label. In

homogeneous assays, the interaction does affect the activity of the label, and therefore analyte levels can be deduced without the need for a separation step.

In general, a target-binding molecule of the present invention may be used diagnostically in the same way that a target-binding antibody is used. Thus, depending on the assay format, it may be used to assay the target, or by competitive inhibition, other substances which bind the target. The sample will normally be a biological fluid, such as blood, urine, lymph, semen, milk, or cerebrospinal fluid, or a fraction or derivative thereof, or a biological tissue, in the form of, e.g., a tissue section or homogenate. However, the sample conceivably could be (or derived from) a food or beverage, a pharmaceutical or diagnostic composition, soil, or surface or ground water. If a biological fluid or tissue, it may be taken from a human or other mammal, vertebrate or animal, or from a plant. The preferred sample is blood, or a fraction or derivative thereof.

In one embodiment, the binding molecule is insolubilized by coupling it to a macromolecular support, and target in the sample is allowed to compete with a known quantity of a labeled or specifically labelable target analogue. (The conjugate of the binding molecule to a macromolecular support is another diagnostic agent within the present invention.) The "target analogue" is a molecule capable of competing with target for binding to the binding molecule, and the term is intended to include target itself. It may be labeled already, or it may be labeled subsequently by specifically binding the label to a moiety differentiating the target analogue from authentic target. The solid and liquid phases are separated, and the labeled target analogue in one phase is quantified. The higher the level of target analogue in the solid phase, i.e., sticking to the binding molecule, the lower the level of target analyte in the sample.

In a "sandwich assay", both an insolubilized target-binding molecule, and a labeled target-binding molecule are employed. The target analyte is captured by the insolubilized target-binding molecule and is tagged by the labeled target-

binding molecule, forming a tertiary complex. The reagents may be added to the sample in either order, or simultaneously. The target-binding molecules may be the same or different, and only one need be a target-binding molecule according to the present invention (the other may be, e.g., an antibody or a specific binding fragment thereof). The amount of labeled target-binding molecule in the tertiary complex is directly proportional to the amount of target analyte in the sample.

The two embodiments described above are both heterogeneous assays. However, homogeneous assays are conceivable. The key is that the label be affected by whether or not the complex is formed.

A label may be conjugated, directly or indirectly (e.g., through a labeled anti-target-binding molecule antibody), covalently (e.g., with SPDP) or noncovalently, to the target-binding molecule, to produce a diagnostic reagent. Similarly, the target binding molecule may be conjugated to a solid-phase support to form a solid phase ("capture") diagnostic reagent. Suitable supports include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to its target. Thus the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc.

#### In Vivo Diagnostic Uses

Analyte-binding molecules can be used for *in vivo* imaging.

Radio-labelled binding molecule may be administered to the human or animal subject. Administration is typically by injection, e.g., intravenous or arterial or other means of administration in a quantity sufficient to permit subsequent dynamic and/or static imaging using suitable radio-detecting

devices. The preferred dosage is the smallest amount capable of providing a diagnostically effective image, and may be determined by means conventional in the art, using known radio-imaging agents as a guide.

5 Typically, the imaging is carried out on the whole body of the subject, or on that portion of the body or organ relevant to the condition or disease under study. The radio-labelled binding molecule has accumulated. The amount of radio-labelled binding molecule accumulated at a given point  
10 in time in relevant target organs can then be quantified.

A particularly suitable radio-detecting device is a scintillation camera, such as a gamma camera. A scintillation camera is a stationary device that can be used to image distribution of radio-labelled binding molecule. The detection  
15 device in the camera senses the radioactive decay, the distribution of which can be recorded. Data produced by the imaging system can be digitized. The digitized information can be analyzed over time discontinuously or continuously. The digitized data can be processed to produce images, called  
20 frames, of the pattern of uptake of the radio-labelled binding protein in the target organ at a discrete point in time. In most continuous (dynamic) studies, quantitative data is obtained by observing changes in distributions of radioactive decay in target organs over time. In other words, a time-  
25 activity analysis of the data will illustrate uptake through clearance of the radio-labelled binding molecule by the target organs with time.

Various factors should be taken into consideration in selecting an appropriate radioisotope. The radioisotope must  
30 be selected with a view to obtaining good quality resolution upon imaging, should be safe for diagnostic use in humans and animals, and should preferably have a short physical half-life so as to decrease the amount of radiation received by the body. The radioisotope used should preferably be pharmacologically  
35 inert, and, in the quantities administered, should not have any substantial physiological effect.

The binding molecule may be radio-labelled with different isotopes of iodine, for example  $^{123}\text{I}$ ,  $^{125}\text{I}$ , or  $^{131}\text{I}$  (see for

example, U.S. Patent 4,609,725). The extent of radio-labeling must, however be monitored, since it will affect the calculations made based on the imaging results (i.e. a diiodinated binding molecule will result in twice the radiation  
5 count of a similar monoiodinated binding molecule over the same time frame).

In applications to human subjects, it may be desirable to use radioisotopes other than  $^{125}\text{I}$  for labelling in order to decrease the total dosimetry exposure of the human body and to  
10 optimize the detectability of the labelled molecule (though this radioisotope can be used if circumstances require). Ready availability for clinical use is also a factor. Accordingly, for human applications, preferred radio-labels are for example,  $^{99\text{m}}\text{Tc}$ ,  $^{67}\text{Ga}$ ,  $^{68}\text{Ga}$ ,  $^{90}\text{Y}$ ,  $^{111}\text{In}$ ,  $^{113\text{m}}\text{In}$ ,  $^{123}\text{I}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$  or  $^{211}\text{At}$ .

15 The radio-labelled binding molecule may be prepared by various methods. These include radio-halogenation by the chloramine - T method or the lactoperoxidase method and subsequent purification by HPLC (high pressure liquid chromatography), for example as described by J. Gutkowska et  
20 al in "Endocrinology and Metabolism Clinics of America: (1987) 16 (1):183. Other known method of radio-labelling can be used, such as IODOBEADS™.

There are a number of different methods of delivering the radio-labelled binding molecule to the end-user. It may be  
25 administered by any means that enables the active agent to reach the agent's site of action in the body of a mammal. If the molecule is digestible when administered orally, parenteral administration, e.g., intravenous, subcutaneous, or intramuscular, would ordinarily be used to optimize absorption.

### 30 Other Uses

The binding molecules of the present invention may also be used to purify target from a fluid, e.g., blood. For this purpose, the target-binding molecule is preferably immobilized on a solid-phase support. Such supports include those already  
35 mentioned as useful in preparing solid phase diagnostic reagents.

Peptides, in general, can be used as molecular weight

markers for reference in the separation or purification of peptides by electrophoresis or chromatography. In many instances, peptides may need to be denatured to serve as molecular weight markers. A second general utility for

5 peptides is the use of hydrolyzed peptides as a nutrient source. Hydrolyzed peptide are commonly used as a growth media component for culturing microorganisms, as well as a food ingredient for human consumption. Enzymatic or acid hydrolysis is normally carried out either to completion, resulting in free

10 amino acids, or partially, to generate both peptides and amino acids. However, unlike acid hydrolysis, enzymatic hydrolysis (proteolysis) does not remove non-amino acid functional groups that may be present. Peptides may also be used to increase the viscosity of a solution.

15 The peptides of the present invention may be used for any of the foregoing purposes, as well as for therapeutic and diagnostic purposes as discussed further earlier in this specification.



## EXAMPLES

## Example 1

*Initial Studies Relating to the Estrogen Receptor*

The estrogen receptor (ER) is a member of the steroid family of nuclear receptors. Like other nuclear receptors, the ER is a ligand dependent transcriptional activator. R. C. J. Ribeiro, P. J. Kushner, J. D. Baxter, *Ann. Rev. Med.* **46**, 443, (1995); J.-M. Wurtz et al., *Nat. Struct. Biol.* **3**, 87 (1996); D. Moras and H. Gronemeyer, *Curr. Opin. Cell Biol.* **10**, 384 (1998). Two distinct estrogen receptors have been described, ER  $\alpha$  and ER  $\beta$ , which may play distinct roles in gene regulation. K. Paech et al., *Science* **277**, 1508 (1997); G. G. J. M. Kuiper and J.-Å Gustafsson, *FEBS Lett.* **410**, 87 (1997); J. T. Moore et al., *Biochem. Biophys. Res. Comm.* **247**, 75 (1998); V. Giguère, A. Tremblay, G. B. Tremblay, *Steroids* **63**, 335 (1998). In addition to the natural ligand, estradiol, the activity of the estrogen receptor is regulated by the association/dissociation of accessory proteins collectively termed co-activators and co-repressors. J. Torchia et al., *Nature* **387**, 677 (1997); C. K. Glass, D. W. Rose, M. G. Rosenfeld, *Curr. Opin. Cell Biol.* **9**, 222 (1997); J. Torchia, C. Glass, M. G. Rosenfeld, *ibid.* **10**, 373 (1998). Upon binding estradiol, the ER undergoes a conformational change that exposes sites for the association of co-activating proteins. This change may also conceal the binding sites for co-repressors or other molecules that are associated with the inactive receptor, thus preventing their association.

The estrogen receptor is a therapeutic target for diseases such as breast and ovarian cancer, and it is also the target for drugs that ameliorate symptoms and effects of menopause including osteoporosis. While effective, compounds that target the estrogen receptor can exhibit a variety of effects in different target tissues. For example, tamoxifen is an estrogen receptor antagonist in breast tissue and is effective in slowing the growth of ER positive breast tumors. However, tamoxifen can have agonist effects on uterine cell growth. M. A. Gallo and D. Kaufman, *Seminars in Oncology* **24** (suppl.1), S1-71 (1997). Because of their wide range of

effects, estrogen receptor targeted drugs cannot be classified as strict agonists or antagonists, but are more appropriately called selective estrogen receptor modulators or SERMS. H. U. Bryant and W. H. Dere, *Proc. Soc. Exp. Biol. Med.* 217, 45  
5 (1998). SERMs appear to drive the receptor into conformations that are neither fully active nor inactive. Distinguishing between these various intermediate conformations in an *in vitro* environment has been a difficult task at best. We have developed peptidic probes that allow distinction between ER  
10 conformations induced by different SERMs. Each SERM, which has a distinct biological effect, also produces a unique pattern in the fingerprint assay. These probes should provide valuable tools for both research and drug discovery, and may provide a link between receptor conformation and biological  
15 activity.

In this example, peptides were identified which bind to the unliganded estrogen receptor  $\alpha$  (ex. 1.1; table 1) or to the estradiol-activated receptor (ex 1.2, table 2). These  $ER\alpha$ -binding peptides were then classified (ex 1.3) into five  
20 arbitrary classes on the basis of their ability to bind to  $ER\alpha$  or  $ER\beta$  in the presence or absence of estradiol. (Naturally, they all bound either the apo- $ER\alpha$  or the estradiol-activated  $ER\alpha$ .) Finally, representative peptides of each class were used to "fingerprint" the known ER SERMs estradiol, estriol,  
25 nafoxidine, tamoxifen or clomifene (ex. 1.4).

*Example 1.1: Identification of peptides that bind to the unliganded (unactivated) estrogen receptor  $\alpha$*

ER alpha (Panvera Corp.) was immobilized on Immulon 4 plastic plates (Dynatech) for the phage affinity selection as  
30 described in patent application Fowlkes, 09/050,359. Peptide sequences obtained for binding to the unliganded (unactivated) receptor are listed below (Table 1).

*Example 1.2: Identification of peptides that bind to the estradiol activated estrogen receptor  $\alpha$*

35 Estrogen receptor was immobilized as described above and incubated with 100  $\mu$ M estradiol for 15 minutes prior to the

addition of phage for affinity selection. Sequences obtained in the presence of estradiol are listed in Table 2.

In the presence of estradiol, numerous sequences were isolated which contain the consensus LXXLL. This motif, which is found in nuclear receptor co-activators, has previously been shown to be necessary and sufficient for their association with nuclear receptors. This association is accomplished via a helical region found in the ligand binding domain of the ER that is exposed upon binding of estradiol. Crystallographic studies indicated that this region is not properly positioned in the presence of some SERMS, thus preventing co-activator association at this site. See generally D. M. Heery, E. Kalkhoven, S. Hoare, M. G. Parker, *Nature* **387**, 73 (1997); M. Nichols, J. M. J. Rientjes, A. F. Stewart, *EMBO J.* **17**, 765 (1998); W. Feng et al., *Science* **280**, 1747 (1998); A. M. Brzozowski et al., *Nature* **389**, 753 (1997).

Consistent with this, peptide sequences containing the LXXLL motif were not isolated during affinity selection on the apo-receptor or in the presence of 4-OH tamoxifen.

*Example 1.3: Classification of peptide sequences*

a.) Comparison of phage vis-a-vis binding to the ER  $\alpha$  and  $\beta$  in the presence or absence of estradiol

Phage expressing distinct peptide sequences were classified according to a number of different parameters. Initial studies measured the relative binding of each of the phage to ER  $\alpha$  and  $\beta$  in the absence or presence of estradiol. ER  $\alpha$  and  $\beta$  were immobilized on Immulon 4 plates and treated for 15 minutes with 100  $\mu$ M estradiol or buffer alone prior to the addition of phage supernatant from a fresh overnight culture. Bound phage were detected using an anti-M13 antibody coupled to HRP. From these results, 12 phage were selected for further study. Sequences were selected that bound preferentially to ER  $\alpha$  or ER  $\beta$  and that bound preferentially in the absence or presence of estradiol.

b) Competition of phage with a peptide containing an LXXLL motif.

The co-activating proteins that have been identified

to date, interact with nuclear receptors via a leucine rich region on the coactivator with the consensus LXXLL, where L is leucine and X is any amino acid. Co-activators containing this consensus motif bind to the ER at helix 12 in the AF2 domain  
5 (the C-terminal transactivation domain). This helical region is exposed when the receptor is activated. Many of the peptide sequences that were isolated for the activated receptor were leucine rich and a great number contained the LXXLL motif. All of these sequences bound preferentially to the activated ER.  
10 A peptide containing an LXXLL motif was synthesized and used in competition assays with phage to determine if the binding of the LXXLL peptide to the ER would affect the binding of the phage. The peptide sequence corresponds to peptide #4 that was isolated in the presence of estradiol: SSNHQSSRLIELLSRSGSGK-  
15 biotin.

ER  $\alpha$  and  $\beta$  were immobilized as described above and pre-incubated in the presence of 100  $\mu$ M LXXLL peptide, 100  $\mu$ M estradiol, buffer alone, or a combination of 100  $\mu$ M estradiol and 100  $\mu$ M peptide for 20 min prior to adding phage supernatant  
20 from a fresh overnight culture. Bound phage were detected as described above. All of the phage expressing an LXXLL containing peptide were competed by the peptide, and several other phage that do not contain the LXXLL motif were also competed by the peptide. These phage may express sequences that  
25 mimic the LXXLL motif, or they may be allosterically affected by the binding of the peptide. There were also phage that do not contain an LXXLL motif that did not compete with peptide.

Based on these data, the peptide sequences were divided into 5 classes listed below, as seen in Table 3 and 4. Table  
30 3 lists peptides of each class, while Table 4 defines the classes. In a comparison of binding to unliganded ER  $\alpha$  and  $\beta$ , class 1 and class 5 peptides have higher affinity for ER  $\beta$ . Class 2, 3 and 4 peptides have higher affinity for ER  $\alpha$ . Ligand (estradiol) increases the affinity of class 1 peptides  
35 for both ER  $\alpha$  and  $\beta$ , and decreases the binding of class 5 peptides to both receptors. Ligand has no effect on the binding of class 2 peptides to either receptor. Ligand increases the binding of class 3 peptides to ER  $\alpha$ , while having

no effect on ER  $\beta$ , and ligand decreases the binding of class 4 peptides to ER  $\alpha$  while also having no effect on ER  $\beta$ . A peptide containing an LXXLL motif, described above, was able to compete with phage from class 1 on both ER  $\alpha$  and  $\beta$ , and with  
5 phage from classes 4 and 5 on ER  $\alpha$  only. Phage from classes 2 and 3 did not compete with the LXXLL peptide on either receptor.

*Example 1.4: Fingerprinting estrogen receptor agonists and SERMs*

10 There are many known agonists and SERMs for the estrogen receptor. For initial testing of the fingerprinting system, two agonists, 17- $\beta$  estradiol and estriol, and three SERMs, 4-OH tamoxifen, nafoxidine and clomiphene, were selected. All three SERMs are derivatives of triphenylethylene. All reagents were  
15 purchased from Sigma.

The effect of agonists and SERMs on the binding of phage from each of the 5 classes described above was investigated. To do this, immobilized ER  $\alpha$  or ER  $\beta$  was incubated with 100  $\mu$ M estradiol, estriol, nafoxidine, tamoxifen or clomifene in TBST  
20 or with TBST alone for 20 minutes prior to adding the phage supernatant from a fresh overnight culture. Following a 1 hour incubation, the wells were washed five times with TBST and the bound phage were visualized using an anti-M13 antibody coupled to HRP.

25 The following fingerprints were identified (Table 6). The data are based on the relative change in binding (as determined by an increase or decrease in absorbance) compared to the unliganded receptor. The number of + or - signs indicates the degree and the direction of the change in signal; +/- indicates  
30 no significant change.

The agonists (estradiol and estriol) produce fingerprints that are distinct from those of the SERMs (tamoxifen, nafoxidine and clomiphene). In addition, the fingerprints are different for ER  $\alpha$  and ER  $\beta$ . As predicted, the agonists, which  
35 have similar biological effects, produce fingerprints that are similar on each receptor. The SERMs are all from the same class of triphenylethylene derivatives and have similar yet

distinct biological effects. The fingerprinting analysis readily distinguishes them from pure agonists and also indicates that they may have similar yet distinct *in vivo* activities.

5        If an increase in the binding of a class 1 peptide indicates agonist activity, then the fingerprint suggests that tamoxifen produces low levels of agonist activity on ER  $\alpha$  and no agonist activity on ER  $\beta$ . Similarly, if the reduction in  
10        the binding of a class 4 peptide indicates agonist activity, then the fingerprint suggests that tamoxifen has antagonist activity on both ER  $\alpha$  and  $\beta$ . The combination of the signals with each peptide class creates a fingerprint for the SERM that provides information on the relative levels of agonist and antagonist activity it produces. The differential changes in  
15        the signals on ER  $\alpha$  and ER  $\beta$  may indicate the tissue specificity of the alteration in receptor activity in response to the SERM.

*Example 2 Further Investigations with Estrogen Receptors*

Affinity selection of phage displayed peptide libraries (Sparks, et al. (1996), Phage Display of Peptides and Proteins, A Laboratory Manual, pp. 227-253) was conducted on both ER $\alpha$  and  
5  $\beta$  under conditions that were predicted to place the ER in different conformations: apo-ER, estradiol bound ER and 4-OH tamoxifen bound ER. Unique sets of high affinity peptides were identified under each condition. Most notably, affinity selection of peptides in the presence of estradiol revealed a  
10 number of sequences containing an LXXLL motif (Table 100A). This motif, which is found in nuclear receptor co-activators (Table 100B), has been shown to be necessary and sufficient for their association with nuclear receptors (Heery, et al. (1997), Nature, 387:733-736). Studies have shown that the association  
15 of the LXXLL motif with the ER is accomplished via a helical region in the ligand binding domain of the receptor that is exposed upon binding estradiol. Structural studies using X-ray crystallography have shown that this region is not properly positioned in the presence of raloxifene (Brzozowski, et al. (1997)) or 4-OH tamoxifen (Shiau, et al. (1998)), thus  
20 preventing the interaction of the co-activator LXXLL motif. The identification of these sequences in the presence of estradiol indicate that the ER is undergoing conformational changes in response to ligand in vitro consistent with the  
25 changes that are predicted to occur in vivo.

*Materials*

Estrogen receptor  $\alpha$  and  $\beta$  were purchased from PanVera Corporation, Madison, WI. Immulon 4 96-well plates were from Dynatech. Streptavidin, 17- $\beta$  estradiol, 4-OH tamoxifen,  
30 nafoxidine, clomiphene, diethylstilbestrol, progesterone, 16- $\alpha$  OH estrone, and estriol were purchased from Sigma. Premarin is a product of Wyeth-Ayerst. Raloxifene is a product of Eli Lilly Corporation. ICI 182,780 was purchased from Tocris Cookson Inc., Ballwin, MO. Anti-M13 antisera was purchased  
35 from Pharmacia. Sequencing of single strand M13 DNA was conducted by Sequetech Corp., Mountain View, CA. Peptide

synthesis was conducted by AnaSpec, San Jose, CA.

*Example 2.1:*

Additional phage affinity selections were made of peptides which bound plastic-immobilized ER $\alpha$  in the presence of the  
5 SERMs 4-OH Tamoxifen, ICI 182,780, or both simultaneously (see Table 7).

*Example 2.2:*

Further phage affinity selections were made with ER $\alpha$  or ER $\beta$  conjugated to ERE (estrogen response element), which in  
10 turn was immobilized. For ER $\alpha$ , selections were carried out with no ligand present (apo-receptor), or in the presence of 17- $\beta$  estradiol, 4-OH Tamoxifen, Raloxifen, or ICI 182,780.

The methodology is described in more detail below. Affinity selection of phage for the various conformations of  
15 the estrogen receptor was conducted essentially as described (Sparks, et al. (1996)). Selections were conducted with the estrogen receptor in TBST (10nM Tris-HCl, pH 8.0, 150 nM NaCl, 0.05% Tween 20), or in TBST containing 1  $\mu$ M 17- $\beta$  estradiol, or 4-OH tamoxifen. Immulon 4 96-well plates were coated with  
20 streptavidin in 0.1 sodium bicarbonate. The plates were then incubated for 1 h with 2 pmol biotinylated, vitellogenin estrogen response element (ERE) per well (Anderson (1998), Biochemistry, 37:17287-17298), followed by incubation for 1 h with 3 pmol (monomer) ER $\alpha$  or ER $\beta$  per well. Oligonucleotides  
25 corresponding to the vitellogenin ERE, biotin-GATCTAGGTCACAGTGACCTGCG (forward) and biotin-GATCCGCAGGTCAGTGTGACCTA (reverse), were synthesized by Genosys. The sequenced active peptides are shown in Table 8. For ER $\beta$ ,  
30 selections were carried out with no ligand present, or in the presence of estradiol or tamoxifen. The resulting active peptides are shown in Table 9.

*Example 2.3*

All of the phage were classified based on their ability



to bind to ER  $\alpha$  and ER  $\beta$ , in the presence or absence of SERMs. These assays were conducted by phage ELISA. In essence, plastic plates were coated with streptavidin (sigma). Biotinylated-EREs (see above) were conjugated to the solid-phase streptavidin, and ER to the ERE. Bound phage were detected using horseradish peroxidase-labeled anti-(M13 phage) antibodies.

The ER was then incubated with 100  $\mu$ l TBST or TBST containing 1  $\mu$ M of the appropriate modulator. Phage (40  $\mu$ l), from a 5 hour culture grown in DH5 $\alpha$ F' cells, was added directly to the wells and incubated 30 minutes at room temperature. Unbound phage were then removed by 5 washes with TBST. Bound phage were detected using an anti-M13 antibody coupled to horseradish peroxidase (HRP). Assays were developed with 2,2'-azinobis(3-ethylbenzothiazoline)-6 sulfonic acid (ABTS) and hydrogen peroxide for 10 minutes and then stopped by the addition of 1% SDS. Absorbance was measured at 405 nm in a Molecular Devices microplate reader.

The results are shown in Tables 11-13, as follows:

- Table 11, Binding of ER $\alpha$ -Selected Peptides to ER $\alpha$  Receptor;
- Table 12, Binding of ER $\alpha$ -Selected Peptides to ER $\beta$  Receptor;
- Table 13, Binding of ER $\beta$ -Selected Peptides to ER $\alpha$  or ER $\beta$  Receptors.

The binding activity is indicated on a semiquantitative scale of 0 to .7+.

#### Example 2.4

##### Selection and Characterization of Panel Peptides

All of the affinity selected phage were evaluated by phage ELISA for binding to apo- ER $\alpha$  and  $\beta$ , and to ER $\alpha$  and  $\beta$  in the presence of estradiol or 4-OH tamoxifen as described above. Many phage showed distinct preferential binding. Some

sequences bound more strongly to the apo-receptor, while others exhibited preferential binding to the estradiol activated or the 4-OH tamoxifen activated receptor. Based on this analysis, eleven phage expressing different peptide sequences and showing distinct binding preferences, were chosen for further use as conformational probes.

Five of these probes bound to both ER  $\alpha$  and ER  $\beta$  ( $\alpha/\beta$  I-V), three were specific for ER  $\alpha$  ( $\alpha$  I-III), and three were specific for ER  $\beta$  ( $\beta$  I-III) (see Table 10). One may view this either as defining a three class panel, with several representatives in each class, or as an eleven class panel, with one member per class. The identification of distinct classes of peptides, some of which recognized both ER $\alpha$  and ER $\beta$ , and others that were receptor specific is consistent with the primary structures of the two receptors being similar yet distinct.

The binding sites of the probes,  $\alpha/\beta$  I-V and  $\alpha$  I-III, were mapped on ER  $\alpha$  using ER $\alpha$  ligand binding domain (residues 282-595) fused to glutathione-S-transferase (GST), an ER $\alpha$  amino terminal domain (1-184) fused to GST, and the full length ER. Assays were conducted using the format described in Example 2.3, except that the domains were directly immobilized on the plastic surface of the well. Assays were conducted as for phage ELISA (Ex. 2.3). Results are shown in Fig. 2.

All of the probes except  $\alpha$  I bound to the ligand binding domain. The  $\alpha$  I probe, which binds only to the full length protein, may be binding to a site that is created by the tertiary structure formed by the interaction between receptor domains.

The probes were used to fingerprint the interaction of ER  $\alpha$  and ER  $\beta$  with a variety of different SERMs by the assay method previously described (using ERE). Next, we evaluated the binding of each of the probes to ER $\alpha$  and ER $\beta$  in the presence of a variety of ER ligands that have distinct biological activities. The goal was to determine if each of the ligands would induce a conformational change in the ER that would alter the binding pattern of the probes, thus producing a "fingerprint" for each compound. The ligands used for this

study include the ER agonists estradiol, estriol, and diethylstilbestrol (DES); the SERMs 4-OH tamoxifen, nafoxidine, clomiphene, and raloxifene; the antagonist ICI 182,780; and the estradiol metabolite 16- $\alpha$ -OH estrone. Premarin, the mixture  
5 of conjugated estrogens used as estrogen replacement therapy, was also included, but it should be noted that many of the components of Premarin must be metabolically activated. Thus, their action may not be detected in this *in vitro* assay. Buffer only (apo-receptor) and progesterone were included as  
10 controls. Information on the structures and biological effects of the SERMs used in this study may be found in the following papers and reviews: B. S. Katzenellenbogen, M. M. Montano, K. Ekena, M. E. Herman, E. M. McInerney, *Breast Can. Res. Treat.* **44**, 23 (1997); J. I. Macgregor and V. C. Jordan, *Pharmacological*  
15 *Rev.* **50**, 151 (1998); B. T. Zhu and A. H. Conney, *Carcinogenesis* **19**, 1 (1998); M. T. R. Subbiah, *Proc. Soc. Exp. Biol. Med.* **217**, 23 (1998); Sulistiyani, S. J. Adelman, A. Chandrasekaran, J. Jayo, R.W. St. Clair, *Arteriosclerosis, Thrombosis, and Vascular Biology* **15**, 837 (1995); B. R. Bhavnani  
20 and A. Cecutti, *J. Clin. Endocrinol. And Metab.* **78**, 197 (1994); B. Bhavnani, *Proc. Soc. Exp. Biol. Med.* **217**, 6 (1998); T.A. Grese et al., *Proc. Natl. Acad. Sci. USA* **94**, 14105 (1997); T. A. Grese et al., *J. Med. Chem.* **41**, 1272 (1998); A Howell, *Oncology* **11**, suppl 1, 59 (1997).

25 As shown in Table 14, each of the ligands tested did indeed alter the binding pattern of the probes, producing a distinct fingerprint for each, whereas the pattern produced by progesterone was indistinguishable from that produced by buffer.

30 The unique ligand dependent binding patterns of the probes indicates that each ligand induces a receptor conformational change that exposes different peptide binding surfaces. The binding patterns for estradiol and ICI 182,780 are distinct or both ER $\alpha$  and  $\beta$ , confirming the conformational change  
35 illustrated by the earlier protease digestion studies. The protease digestion assay, which relies on the location of cleavage sites for detection of conformational changes, could distinguish between conformational changes induced by estradiol

and 4-OH tamoxifen or estradiol and ICI 182,780. However, it was unable to distinguish between changes induced by 4-OH tamoxifen and other ER modulators such as ICI 182,780. The fingerprint assay, however, clearly indicates that unique peptide binding surfaces are exposed on both ER $\alpha$  and  $\beta$  in the presence of 4-OH tamoxifen that are not exposed in the presence of ICI 182,780. Tamoxifen, nafoxidine and clomiphene contain the same triphenylethylene core structure. These three compounds, although similar in structure, produce distinct biological effects. Therefore, it might be predicted that these compounds would induce similar, yet distinct, conformational changes in the receptors. The fingerprint assay shows that the probes  $\alpha/\beta$  III, IV and V, which have high affinity for the ER in the presence of 4-OH tamoxifen, have lower affinity for the ER complexed with nafoxidine and clomiphene, indicating that these peptide binding surfaces differ in the presence of these compounds. The  $\alpha$  III probe more clearly differentiates these three compounds. The fingerprint assay also differentiates 4-OH tamoxifen and raloxifene. The probes  $\alpha/\beta$  III, IV and V have reduced affinity for both ER  $\alpha$  and  $\beta$  in the presence of raloxifene compared to 4-OH tamoxifen. The probes  $\alpha/\beta$  II,  $\beta$  I and  $\beta$  III further distinguish ER  $\beta$  conformational changes induced by these two compounds. The fingerprint pattern produced by Premarin is distinct compared to other agonists; however, Premarin's activities are due to a mixture of components. It would be interesting to assess the binding patterns of the probes in the presence of each of the purified, activated components of Premarin.

The probe  $\alpha/\beta$  I contains an LXXLL motif. The binding of estradiol to the ER strongly enhanced the binding of this probe to both ER  $\alpha$  and ER  $\beta$ . However, estriol, Premarin and DES, which are also considered ER agonists failed to activate the binding of this probe to ER  $\alpha$  to the same extent as estradiol. On ER  $\beta$ , the binding of the probe was enhanced significantly with all of the agonists. The SERMs, 4-OH tamoxifen, nafoxidine, clomiphene, raloxifene and ICI 182,780 prevented the binding of this probe to both ER  $\alpha$  and  $\beta$  and appeared to

reduce the binding to a level below that which is observed in buffer alone.

The probes  $\alpha/\beta$  III-V show enhanced binding in the presence of SERMs, particularly 4-OH tamoxifen, indicating that a new binding surface is exposed on the ER in the presence of these compounds. The binding patterns of these three probes along with the probes  $\alpha/\beta$  II,  $\alpha$  III,  $\beta$  I and  $\beta$  III illustrate differences in the receptor conformation induced by 4-OH tamoxifen, nafoxidine, clomiphene, and raloxifene. Since the binding of the probes to the ER in the presence of these SERMs may be altered but not abrogated, subtle changes in receptor conformation can be visualized. **This is the first *in vitro* assay that distinguishes between these four compounds.** The probe  $\alpha$  II is also unique in that it binds to ER  $\alpha$  in the presence of any compound that binds to the estrogen receptor, indicating that while some receptor conformational changes are unique to the modulator, others may be more universal. Overall, these probes allow the detection of both subtle and distinct conformational changes that are induced by many different modulators of ER activity.

To confirm that the binding of the probes to the ER was dependent upon the peptide expressed on the surface of the phage, biotinylated peptides corresponding to the sequences were synthesized with biotin attached to a carboxy-terminal lysine. The peptides were coupled to europium labeled streptavidin and binding studies were conducted using time resolved fluorescence spectroscopy (TRF).

Time resolved fluorescence (TRF) assays were performed at room temperature as follows: Costar high-binding 384 well plates were coated with streptavidin in 0.1 M sodium bicarbonate and blocked with bovine serum albumin. Biotinylated ERE (2 pmol) was added to each well. Following a 1 h incubation, biotin was added to check any remaining binding sites. The plates were washed and 2 pmol ER $\alpha$  was added to each well. Following a 1h incubation, the plates were washed and the ER modulators were added at a range of concentrations, from picomolar to micromolar. Following a 30

min incubation with the modulators, 2 pmol of a europium labeled streptavidin (Wallace)-biotinylated peptide conjugate (prepared as described below) was added and incubated for 1 h. The plates were then washed and the europium enhancement  
5 solution was added. Fluorescent readings were obtained with a POLARstar fluorimeter (BMG Lab Technologies) using a <400 nm excitation filter and a 620 nm emission filter. The europium labeled streptavidin-biotinylated peptide conjugate was prepared by adding 8 pmol biotinylated peptide to 2 pmol  
10 labeled streptavidin. After incubation on ice for 30 min, the remaining biotin binding sites were blocked with biotin prior to addition to the ER coated plate.

The binding of the probes to the ER was measured. The results, shown in Table 15, indicate that the peptides are  
15 indeed conferring the binding specificity. Comparison of the fluorescence values obtained from the TRF binding assays and the signals obtained in the phage ELISA fingerprint indicates that the two methods produce similar patterns. However, the binding assay also provides an indication of the potency of  
20 each compound to induce the conformational change required for peptide binding. Taken together, these results indicate that conversion of the fingerprint assay from phage to peptides will provide an even more sensitive assay for detecting conformational change.

25 One of the most notable observations from the TRF binding assays is that the binding of the  $\beta$  I probe to ER  $\beta$  is enhanced in the presence of the SERM 4-OH tamoxifen and reduced in the presence of other SERMs such as raloxifene, nafoxidine, and clomiphene. The reduction in binding observed with these  
30 compounds is similar to the reduction observed with agonists such as estradiol, estriol, and DES.

We have identified peptides that serve as conformational probes of the estrogen receptor  $\alpha$  and  $\beta$ . Many probes bind to both receptors, while other probes bind preferentially to  
35 either the  $\alpha$  or  $\beta$  receptor. Consistent with the two receptors having regions of high homology and other more divergent regions, these results indicate that the receptors have some

binding surfaces in common, while others are unique. The implications of this are that both receptors may contact some of the same regulatory proteins in the cell, yet there may be additional proteins that specifically regulate either ER  $\alpha$  or  $\beta$  action.

We have used our peptidic probes to show that both receptors undergo distinct conformational changes as a result of binding different ligands. The probes not only reveal receptor conformational changes by their relative changes in affinity, but they also identify unique binding surfaces on the two receptors. These binding surfaces may, in fact, be the surfaces that interact with various co-regulatory proteins in response to different ligands. For example, many peptides selected with the estradiol activated receptor contained sequences found in nuclear receptor co-activators, as illustrated by the peptides containing the LXXLL motif (Figure 1). These peptide probes are probably mimicking the interaction between the receptor and co-activating proteins. Potentially, these probes can be used to identify heretofore, unknown receptor-protein interactions.

Additional applications of the probes lie in the area of detection of ER modulators. One or more probes can be used to set up a high-throughput screen to identify modulators of ER activity. We anticipate that compounds that bind to the ER will alter receptor conformation and hence alter the binding patterns of the probes. The sites targeted by the screen may not be *bona fide* protein-protein interaction surfaces, but may represent sites exposed in the presence of a specific ligand, and thus serve as markers for specific conformations. The fingerprinting technique may also be applied to quickly classify hits from a screen into different categories such as agonist (resembling the estrogen pattern), antagonist (resembling the ICI 182,780 pattern), mixed (resembling the tamoxifen pattern) or novel effectors, prior to assessing them in a cell-based assay. Fingerprinting may also be used to determine structure activity relationships and to rapidly assess compounds following chemical modification during lead optimization.

- This is the first technique described that can distinguish between estrogen receptor conformations induced by ligands both between and within ligand classes. The data gathered with this assay provide strong evidence that the biological activity of the estrogen receptor can be linked to the conformation induced upon binding ligand. A strength of this fingerprinting technique is that it is broadly applicable to any protein or receptor that undergoes structural changes upon binding of a ligand or substrate.
- These studies confirm that the assays may readily be conducted with synthetic peptides in place of phage-bound and -expressed peptides.

#### *Example 2.5 Analysis of Known SERMS using Panel*

- For fingerprint analysis of estrogen receptor modulators on ER  $\alpha$  and ER  $\beta$ , estrogen receptor (3 pmol) was immobilized on 2 pmol biotinylated ERE. Immobilized ER was incubated with estradiol (1  $\mu$ M), estriol (1  $\mu$ M), premarin (10  $\mu$ M), 4-OH tamoxifen (1  $\mu$ M), nafoxidine (10  $\mu$ M), clomiphene (10  $\mu$ M), raloxifene (1  $\mu$ M), ICI 182,780 (1  $\mu$ M), 16 $\alpha$ -OH estrone (10  $\mu$ M), DES (1  $\mu$ M) or progesterone (1  $\mu$ M) for 5 minutes prior to the addition of phage. Phage were amplified from plaques in DH5 $\alpha$  F' for 5 hours. Bound phage were detected as described previously. Assays were developed with ABTS (2,2'-azinobis(3-ethylbenzthiazoline-sulfonic acid) for 10 minutes.
- The results are shown in Tables 14A and 14B. Table 14A shows binding to the ER $\alpha$  receptor and Table 14B binding to the ER $\beta$  receptor. It is not necessary to list all 11 panel peptides in each table since some only bind the ER $\alpha$  and others only the ER $\beta$ . The binding activity is indicated on a semiquantitative scale of 0 to 7+.

#### *Example 2.6 Calculation of Similarity Between SERMs*

Based on Tables 14A and B, one may define a fingerprint for each SERM. This fingerprint is an array of descriptors, each of which is a value in the Table representing the binding



affinity of a particular panel peptide for either ER $\alpha$  or ER $\beta$  in the presence of the SERM in question. The tables in question allow each fingerprint to be composed of 16 descriptors (one for each row in the tables). We obtain 12 fingerprints, one for each of the 11 SERMs, plus buffer.

We can therefore calculate the similarity of between each of the 12x12=144 possible pairings of these fingerprints. To begin with, we calculate the Euclidean distance between each fingerprint. This is the square root of the sum of the square of the differences between the respective column values in Tables 14A and 14B. For example, the distance between buffer and Estradiol is the square root of the sum of the square of the 16 descriptor pair differences, i.e., the square root of the sum of

15        (1-6)<sup>2</sup>, 25;  
           (7-2)<sup>2</sup>, 25;  
           (1-1)<sup>2</sup>, 0;  
           (1-1)<sup>2</sup>, 0;  
           (1-1)<sup>2</sup>, 0;  
 20        (7-7)<sup>2</sup>, 0;  
           (1-6)<sup>2</sup>, 25;  
           (5-2)<sup>2</sup>, 9;  
           (2-7)<sup>2</sup>, 25;  
           (7-2)<sup>2</sup>, 25;  
 25        (2-1)<sup>2</sup>, 1;  
           (1-1)<sup>2</sup>, 0;  
           (6-3)<sup>2</sup>, 9;  
           (1-5)<sup>2</sup>, 16; and  
           (7-7)<sup>2</sup>, 0;

30        for a total of 160, the square root of which is about 12.65.

The maximum possible distance in the present instance is the square root of  $16 \times (7 \times 7)$ , which is 28. This is because each descriptor pair has a maximum possible difference of 7, and there are 16 descriptors in the fingerprint.

5       The distance may be converted into a similarity by

$$\text{current similarity} = (\text{maximum distance} - \text{current distance}) / \text{maximum distance},$$

which equals 1 when the current distance is 0, and 0 when the current distance equals the maximum distance. In the example  
10   above (buffer:estradiol) the similarity is 0.55.

In contrast, the fingerprints of cloniphene and raloxiphenes are at a Euclidean distance of  $\sqrt{40}$ , which is 6.32, and therefore have a similarity of 0.77.  $16\alpha$ -OH estrone and DES are even more similar, with a Euclidean distance of  
15    $\sqrt{7}$ , which is 2.645, and therefore a similarity of 0.91.

On the other hand, the fingerprints of estradiol and cloniphene are at a Euclidean distance of  $\sqrt{210}$ , which is 14.49. This corresponds to a similarity of 0.48.

It will be appreciated that we could have changed the  
20   choice and/or number of descriptors incorporated into the fingerprint, rescaled and/or weighted the descriptors in some way, used a different measure of distance, and/or converted distance into similarity by another method. We could also have determined similarity without first calculating a  
25   distance.

In the above text, we have lumped together the data for  $ER\alpha$  and  $ER\beta$ . We could have calculated separate fingerprints and similarities for each form of ER. This is shown in Figs. 5 and 6.

30       Thus, for buffer:estradiol, the distance between their fingerprints for  $ER\alpha$  binding is  $\sqrt{84}$ , and for  $ER\beta$  binding,  $\sqrt{76}$ , or 9.16 and 8.72. The maximum distance is  $\sqrt{196}$  ( $14 \times 14$ ), which is 14. So the similarities are 0.54 and 0.56 respectively, which aren't much different.

On the other hand, for ICI 182 780:16 $\alpha$ -OH estrone, we calculate distances of SQRT(6) for ER $\alpha$  binding, and SQRT(76) for ER $\beta$  binding, corresponding to similarities of 0.88 and 0.56, respectively. So these compounds are more similar in how they bind ER $\alpha$  than in how they bind ER $\beta$ .

This fingerprinting technique provides a rapid and sensitive method to detect changes in protein conformation. We have applied this technique to ER  $\alpha$  and  $\beta$  and demonstrated that these two receptors undergo different conformational shifts in response to various modulators of activity. Because the pattern of probe binding is unique for each modulator, the assay can be used to distinguish compounds both between and within modulator classes. The assay can also be used to identify modulators that have specificity for either the  $\alpha$  or  $\beta$  form of the receptor.

One or more probes can be used to set up a high-throughput screen (HTS) to identify modulators of ER activity. Compounds that bind to the ER and alter receptor conformation will alter the binding patterns of the probes. This technique may also be applied to classify hits from a HTS as agonist (resembling the estrogen pattern) antagonist (resembling the ICI 182,780 pattern) or mixed (resembling the tamoxifen pattern) prior to assessing them in a cell-based assay. Fingerprinting may also be used for structure activity relationships. As chemical modifications are made to lead molecules, fingerprinting will provide a convenient method to quickly determine if the modification affects receptor conformation in a manner different than the parent compound.

All of the compounds used in this study are known to produce unique biological effects *in vivo*. Many of the differential effects are tissue specific, perhaps due to differential expression of regulatory proteins and/or the two forms of the receptor. Each of these compounds also produces a unique fingerprint pattern *in vitro*, derived from the conformation adopted by the receptors upon binding the modulator. Thus, fingerprinting conformational changes induced by SERMs *in vitro* is expected to be useful for predicting the

in vivo biological activities of modulators.

*Example 3: Fingerprinting Using Yeast Two-Hybrid Cell-Based Assays*

5       The two hybrid methods of examining protein/protein interactions initially described by Fields and Song (Nature 340:245-246 (1989)) and later by Gyrius, et al (Cell 75:791-803 (1993)) utilize similar technologies. In both cases a yeast cell is provided as the host cell which carries a reporter gene  
10       operated by an upstream protein binding site (DNA binding site). The host cells carry a plasmid expressing peptide/protein fusions with the specific binding protein or domain (DNA binding domain). The host cell also carries a plasmid expressing a peptide/protein fusion with a  
15       transcriptional activation protein or domain (Activation domain). If the two peptide/protein fusions are capable of directed interactions within the cell, transcriptional activation of the reporter gene occurs. The level of reporter gene transcription is reflective of the strength of the  
20       interaction between the two protein fusions.

      The LexA system that we employ utilizes a bacterial DNA binding protein domain, LexA, and a bacterially derived transcriptional activation sequence, B42. Proteins or peptides of interest are fused in frame with these domains and expressed  
25       using episomal plasmids in a yeast cell. The interactions between these proteins/peptides of interest are registered by monitoring the level of the reporter gene product,  $\beta$ -galactosidase, by an enzymatic assay. The differences in the levels of  $\beta$ -galactosidase activities reflect the relative  
30       strengths of the protein interactions.

      We have tested the interactions of peptides F6 (an affinity-selected peptide with a high affinity for ER $\alpha$ ), alpha2 (A2), alpha/beta 3 (AB3), and alpha/beta 5 (AB5) with estrogen receptor  $\alpha$  using the LexA yeast two hybrid system in the  
35       presence of agonist or antagonist. These peptides were isolated previously from phage display libraries using estrogen receptor  $\alpha$  (ER $\alpha$ ) as a target. The interactions between these

peptides and ER $\alpha$  are altered in the presence of agonist or antagonist in the in vitro phage display system. For example, peptide  $\alpha 2$  was found to bind in the presence of estradiol and 4-OH-tamoxifen, but not in their absence; peptide  $\alpha/\beta$  3 binds  
5 to ER $\alpha$  only in the presence of 4-OH-tamoxifen, not estradiol or in the absence of any compound. We undertook the yeast two hybrid analysis to investigate whether these in vitro results could be recapitulated in vivo. The results from the yeast two hybrid system were qualitatively similar to those that were  
10 performed using phage display on purified ER $\alpha$  protein.

#### Yeast strains and genetic manipulations

##### References for plasmids and strain

##### Cloning vector pJG4-5

Genbank Accession number: U89961

- 15       Reference: Gyuris, J., Golemis, E., Chertkov, H. and Brent, R.  
Cdi1, a human G1 and S phase protein phosphatase that  
associates with Cdk2. Cell 75 (4), 791-803 (1993)

##### Cloning vector pEG202 (pLexA), complete sequence.

Genbank Accession number: U89960

- 20       AUTHORS   Golemis, E., Gyuris, J. and Brent, R.  
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##### pJK103

- 25       Reference: J. Kamens and R. Brent A yeast transcription  
assay defines distinct *rel* and *dorsal* DNA recognition  
sequences. New Biol. 3:1005-1013 (1991).

##### Yeast Strain EGY48

- Reference: Gyuris, J., Golemis, E., Chertkov, H. and Brent, R.  
30 Cdi1, a human G1 and S phase protein phosphatase that

associates with Cdk2. Cell 75 (4), 791-803 (1993)

The yeast strains used in this study was EGY48 ( MAT  $\alpha$  trp1 his3 ura3 leu2::6lexAops-LEU2) purchased from OriGeneTechnologies for yeast two hybrid analysis. This strain  
 5 contains 6 LexA operators upstream of the LEU2 gene in the yeast genome and provides high sensitivity in detecting protein-protein interactions in the LexA two hybrid system. The plasmids used in this study were pEG202 (LexA-DNA binding domain), pJG4-5 (B42-activation domain), and the plasmid  
 10 containing a  $\beta$ -galactosidase reporter, pJK103 (OriGene Technologies). The full length estrogen receptor was subcloned in frame into the EcoRI and XhoI sites of pJG4-5 to generate an ER $\alpha$ -B42 activation domain fusion. ER $\alpha$  was subcloned in the activation domain plasmid because ER $\alpha$  was able to autoactivate  
 15 reporters when fused to the LexA DNA binding domain.

The peptide sequences used in this study were generated from synthetic oligos filled in by T7 Sequenase (Life Science) and subcloned into the EcoRI-XhoI sites of pEG202. The synthetic oligos were:

20 F6, 5' -  
 GACTGTGCGAATTCGGTCATGAACCATTAACCTTTATTAGAAAGATTATTAATGGATGATA  
 AACAAAGCTGTTCTCGAGCGTGTCTAG;  
 $\alpha$ II, 5' -  
 GACTGTGCGAATTCCTCTTTAACTTCTAGAGATTTTGGTTCTTGGTATGCTTCTAGAC  
 25 TCGAGCGTGTCTAG;  
 $\alpha/\beta$ III, 5' -  
 GACTGTGCGAATTCCTCTTCTTGGGATATGCATCAATTTTTTTGGGAAGGTGTTTCTAGAC  
 TCGAGCGTGTCTAG;  
 $\alpha/\beta$ V, 5' -  
 30 GACTGTGCGAATTCCTCTCTCCAGGTTCTAGAGAATGGTTTAAAGATATGTTATCTAGAC  
 TCGAGCGTGTCTAG.

The complementary synthetic oligo used to generate double stranded DNA was 3'XhoPrim, 5'-CTGACACGCTCGAG. Each 5'-oligo was annealed to the 3'-oligo by heating to 90 °C for 15 minutes

and cooled slowly to 35 °C. T7 sequenase was added and the fill-in reaction allowed to proceed at 30 °C for 30 minutes. The reaction was terminated by heat denaturing the enzyme at 65 °C for 1 hour, restriction digests were performed and the  
5 resulting DNA fragments subcloned into pEG202 to generate peptide-LexA DNA binding domain fusions.

Yeast cells were transformed by the method of Ito et al. (J. Bacteriol. 153: 163-168 (1983)) and grown on selective media.

10         $\beta$ -galactosidase activity assays

10 ml cultures of yeast strain EGY48 containing pJG4-5 ER $\alpha$  pJK103 and pEG202-F6, - $\alpha$ II, - $\alpha$ / $\beta$ III, or - $\alpha$ / $\beta$  V were grown overnight at 30 °C in selective media containing 100 nM estradiol, 4-OH tamoxifen, or tamoxifen citrate with galactose  
15 as the carbon source. The culture was diluted to  $\sim 2 \times 10^6$  cells/ml in the same media and allowed to grow at 30 °C until the cultures reached a density of  $\sim 1 \times 10^7$  cells/ml ( $\sim 4$  hours). The yeast cells were pelleted by centrifugation, washed with extraction buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM  
20 MgSO<sub>4</sub>, 1 mM PMSF, 7 mM 2-mercaptoethanol) and suspended in 200  $\mu$ l of extraction buffer. 100  $\mu$ l of acid-washed glass beads were added and cells were lysed by vigorous agitation for 10 minutes at 4 °C. Cellular debris was pelleted by centrifugation and the supernatant transferred to a clean tube.  
25 10  $\mu$ g of total cellular protein was diluted into complete Z buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>, 7 mM 2-mercaptoethanol) to a volume of 100  $\mu$ l in a 96-well microplate. 80  $\mu$ g of o-nitrophenyl- $\beta$ -D-pyranoside (ONPG) in 20  $\mu$ l was added to each well to initiate color development.  
30 The reaction was stopped by the addition of 30  $\mu$ l 1M Na<sub>2</sub>CO<sub>3</sub> and the time for development was noted.  $\beta$ -galactosidase activity was determined by measuring the absorbance at 405 nm.

Yeast cultures were grown in the presence or absence of 100nM estradiol, 4-hydroxy-tamoxifen, or tamoxifen citrate and  
35 protein extracts prepared as described in methods. 10 $\mu$ g of each protein extract was assayed for  $\beta$ -galactosidase activity using o-nitrophenyl-b-D-pyranoside as substrate. Activity

units are defied as  $1000 \cdot \text{Abs}_{405} / \text{minutes} / \text{mg} / \text{protein}$ .

The results are shown in Table 99.

5 The peptides that were used in the two hybrid assay were originally isolated by phage display using ER alpha as the target protein. The isolation procedure was carried out in the presence of agonist or antaogonists (estradiol, 4-hydroxy tamoxifen,...) which generated a differing set of interacting peptides. The interactions of these peptides with ER alpha were investigated in vitro using diferent agonists and  
10 antagonists. The interaction profile generated by these in vitro studies allows us to use these peptides as probes for the physical state of the estrogen receptor. The two hybrid assay discerns whether these interactions can be maintained whithin the cell.

15 The results from the two hybrid experiments show a qualitatively similar interaction profile between the peptides and ER alpha as determined in vitro. Therefore, the effects of agonists and antagonists on the structure and availability of peptide binding sites on ER alpha is maintained in vitro and  
20 in vivo. These results allow interpretation of the structural state (activated or antagonized) of ER alpha in response to various compounds. The results using known activators and antagonists can be used to identify other unkown compounds as agonists or antagonists in a drug screen. The availability of  
25 more peptides and use of other known agonists and antagonists will generate better tools for identifying possible compounds or drug leads.



*Example 4 Use of Mammalian Two-Hybrid Assays to Explore ER Activation*

The estrogen receptor (ER) plays an important role in both normal and pathological processes of human development and disease. Clinically, ER antagonists such as tamoxifen have met with much success in the treatment of ER containing breast cancers. However, resistance to tamoxifen usually develops within 2-5 years after initial treatment. A potential mechanism of resistance may be the ability of tumors to switch from recognizing tamoxifen as an antagonist to responding to it as an agonist.

In this regard, several new tissue specific antiestrogens have been developed which may have clinical utility in the treatment of tamoxifen refractory breast cancers.

In an attempt to identify novel high affinity ligands which target the ER/tamoxifen complex, we have employed the use of phage display to screen for random peptides which will recognize the specific ER conformation induced by tamoxifen. We have isolated a series of 15mer peptides which can recognize this complex. Furthermore, these peptides are able to form complexes in vivo with ER as assessed in the mammalian two-hybrid system. Using various ER mutants, we have mapped the peptide interaction surface to the hormone binding domain. Importantly, we have demonstrated that expression of these peptides can block the partial agonist activity of tamoxifen in cells transfected with ER. Although the mechanism by which these peptides block ER/tamoxifen transcriptional activity remains unknown, it appears that DNA binding and ER stability are not affected by peptide expression. Therefore, it is possible that these peptides may be targeting a functionally active site present in the ER/tamoxifen conformation.

This makes possible a novel approach in the development of rational drug design for the treatment of tamoxifen refractory breast cancers. Traditionally, only molecules which interact with the ligand binding pocket have been considered for the development of novel ER antagonists. In addition to these traditional "hormonal" agents, we propose that the

ability to target the specific receptor conformation induced by hormones will result in the development of therapeutically important novel pharmaceuticals. Furthermore, these findings may be applied to other nuclear receptors for which transcriptional interference may be clinically useful.

#### Example 4.1

Figure 7 shows the development of a cell-based assay to assess peptide-receptor interactions. Peptide sequences representing each class was fused to the DNA binding domain (DBD) of the yeast transcription factor Gal4. HepG2 cells were then transiently transfected with expression vectors for ER $\alpha$ -VP16 and the Gal4-peptide fusion proteins. In addition, a luciferase reporter construct under the control of 5 copies of a Gal4 upstream enhancer element was also transfected along with a pCMV- $\beta$  galactosidase vector to normalize for transfection efficiency. Transfection of the Gal4 DBD alone is included as control. Cells were then induced with various ligands as indicated in the figure and assayed for luciferase activity and  $\beta$  galactosidase activity. Normalized response was obtained by dividing the luciferase activity by the  $\beta$  galactosidase activity.

Results. ER $\alpha$  does not interact with the Gal4 DBD alone under any condition.  $\alpha\beta$ I interacts with ER in the presence of estradiol and somewhat with the apo-receptor.  $\alpha$  II interacts with the receptor under all conditions with the apo-receptor and ICI 182,780 bound receptor showing the least activity.  $\alpha\beta$  III and  $\alpha\beta$  V interact almost exclusively with the tamoxifen bound receptor. This data in general confirms that obtained from the time resolved fluorescent study. Furthermore, the ability of these peptides to act as conformational detectors confirms in the cell. earlier observations obtained from protease digestion and crystallization studies that the receptor undergoes distinct conformational changes when bound by different ligands.

#### Example 4.2

The specificity of peptide-nuclear receptor interaction was analyzed (Fig. 8) using the mammalian two-hybrid system. Experimental design is the same as in figure 2 except that either progesterone receptor (PRB-VP16), estrogen receptor beta (ER $\beta$ -VP16), glucocorticoid receptor (GR-VP16) or thyroid receptor beta (TR $\beta$ -VP16) was tested.

Results. All receptors tested, as expected, interact with the  $\alpha\beta$  I peptide in the presence of the appropriate agonist for that receptor. None of the receptors tested interact significantly with the  $\alpha$  II or  $\alpha\beta$  III peptide. This was somewhat surprising considering that  $\alpha\beta$  III was originally isolated on ER $\beta$ . This suggests that the conformation of ER $\beta$  in the cell may be different from that of the purified receptor in vitro. Interestingly,  $\alpha\beta$  V was able to associate with both PRB and ER $\beta$ . This peptide bound ER $\beta$  only in the presence of tamoxifen but was able to associate with PRB in the presence of the PR antagonists RU 486 and ZK 98299. This suggest that  $\alpha\beta$  V is capable of recognizing the antagonist conformation of a subset of nuclear receptor family members.

#### 20 Example 4.3

Figure 9 demonstrates that certain peptides which interact with the tamoxifen activated estrogen receptor do not require AF-2(Helix 12) of the receptor. Three ER $\alpha$  mutants were compared with wild-type ER $\alpha$ . ER-LL was characterized by mutations L540A/L541A. Mutant ER3X was characterized by mutations D538N/E542Q/D545N. Finally, mutant ER-535 STOP was truncated after residue V535. These mutations have been shown to significantly compromise ER AF-2 transcriptional activity and their interaction with several known coactivators. Mutant ER3X is partially AF-2 active and ERL and ER535-stop are AF-2 inactive. Experimental design is the same as in figure 7 except that either (A) ER3X (B) ERL or (C) ER535-stop was analyzed for binding to conformation sensitive peptides.

Results.  $\alpha\beta$  I peptide is unable to interact with the ERL and ER535-stop mutant receptors in the presence of estradiol indicating that these mutations may abolish coactivator

binding.  $\alpha\beta$  I peptide retains some ability to engage the ER3X mutant receptor in the presence of estradiol suggesting that these mutations significantly lower the affinity of the receptor for coactivators but does not destroy this interaction. These findings are consistent with the transcriptional properties of these receptors.  $\alpha$  II peptide binding specificity is largely unaffected by any of the receptor mutations tested. Interestingly, the  $\alpha\beta$  III and  $\alpha\beta$  V peptides specificity of interaction is modified with each successive mutation resulting in a loss of the tamoxifen specificity and resulting in the ability of these peptides to engage the receptor in the presence of many of the ligands tested. These results suggest that although helix 12 is not required for the binding of peptides which recognize the conformation induced by tamoxifen that normal helix 12 structure is required for the specificity of interaction of  $\alpha\beta$  III and  $\alpha\beta$  V peptides.

#### Example 4.4

Figure 10 studies the disruption of ER mediated transcriptional activity by Gal4-peptide fusion proteins. HepG2 cells were transfected with the estrogen responsive C3-Luc reporter gene along with expression vectors for ER $\alpha$  and  $\beta$  galactosidase. Cells were induced with either estradiol or tamoxifen as indicated in the figure and analyzed for luciferase and  $\beta$  galactosidase activity (10A).

Then HepG2 cells were transfected as above except that expression vectors for Gal4-peptide fusions were included as indicated in 10B. Control represents the transcriptional activity of estradiol (10 nM) activated ER in the presence of the Gal4- DBD alone and is set at 100% activity. Increasing amounts of input plasmid for each Gal4-peptide fusion is also shown with the resulting transcriptional activity presented as % activation of control. Data is averaged from three independent experiments (each performed in triplicate) with error bars representing standard error of the mean, subfig. C is same as in (B) except that 4-OH tamoxifen was used to

activate the receptor.

Results. Tamoxifen displays partial agonist activity in HepG2 cells. This activity is up to 30% of that exhibited by estrogen.  $\alpha\beta$  I and  $\alpha$  II peptides are able to inhibit the ability of estradiol to activate transcription up to 50% under the conditions of this assay. It is not surprising that the  $\alpha\beta$  I peptide inhibits ER activity due to the fact that it probably competes for coactivator binding. The ability of  $\alpha$  II peptide to disrupt ER transcriptional activity may suggest that this peptide recognizes some pocket in the receptor that is also important for coactivator binding. The inability of  $\alpha\beta$  III and  $\alpha\beta$  V to block estradiol mediated transcription correlates well with their inability to bind the receptor when bound by estradiol. Interestingly,  $\alpha$  II,  $\alpha\beta$  III and  $\alpha\beta$  V are able to efficiently block the partial agonist activity of tamoxifen while  $\alpha\beta$  I is not. These findings are in agreement with the binding characteristics of these peptides and may suggest that the pocket(s) recognized by these peptides are important for the ability of tamoxifen to behave as a partial agonist.

#### Example 4.5

Figure 11 shows disruption of tamoxifen activated ER transcriptional activity by  $\alpha$ II peptide is not promoter dependent. Experimental design is the same as in figure 6 except that the ability of  $\alpha$ II peptide to inhibit tamoxifen (10nM) activated transcription was tested on several distinct promoters including 1x-ERE-Luc, 3X-ERE-Luc, TK-ERE-Luc and C3-Luc.

Results. The ability of  $\alpha$  II peptide to block tamoxifen activated transcription is not dependent on the context of the promoter. This peptide blocks tamoxifen partial agonist activity from all promoters tested.

#### Example 4.6

Figure 12 shows disruption of ER mediated transcriptional

activity through the AP-1 pathway by Gal4-peptide fusion proteins. (A) HepG2 cells were transfected with the AP-1 responsive collagenase reporter gene construct (pCOL-Luc) and expression vectors for ER $\alpha$  and  $\beta$ -galactosidase. Cells were  
5 then induced with either estradiol or tamoxifen as indicated in the figure and assayed for luciferase and  $\beta$ -galactosidase activity and normalized as detailed in figure 7. (B) Same as (A) except that Gal4-peptide fusion constructs were also transfected as indicated in the figure. **Control** represents the  
10 transcriptional activity of either estradiol or tamoxifen (100nM) activated ER in the presence of the Gal4 DBD alone and is set at 100% activity. The transcriptional activity of estradiol and tamoxifen is shown in the presence of each Gal4-peptide fusion with the resulting transcriptional activity  
15 presented as % activation of control. Data presented is from a single representative experiment.

Results. Both estradiol and tamoxifen are able to activate transcription from the AP-1 responsive collagenase reporter gene. This activity is manifest in the absence of an  
20 estrogen response element (ERE) and is believed to occur through some mechanism involving an interaction between ER and the AP-1 proteins Fos and Jun. As with the C3-Luc reporter gene, each peptide is able to inhibit ER mediated transcriptional activity according to its ability to interact  
25 with the receptor in a ligand dependent manner. Those peptides which interact with the estradiol bound receptor inhibit estradiol mediated transcription while those which interact with the tamoxifen bound receptor inhibit tamoxifen mediated transcription.

#### 30 Example 4.7

Figure 13 model of the potential mechanisms by which peptides block the partial agonist activity of tamoxifen. (A) Model of the activation pathway by which tamoxifen exhibits partial agonist activity. Upon binding tamoxifen (T), the  
35 receptor undergoes a conformational change which allows it to interact with some as yet unidentified coactivator protein.

This protein in turn transmits a signal to the general transcription machinery which results in activation of transcription. (B) In this model of inhibition, the receptor undergoes a conformational change when bound by tamoxifen but  
5 the coactivator protein is unable to engage the receptor due to competition for the same site by the peptide. (C) In this model of inhibition, the receptor undergoes a conformational change in the presence of tamoxifen which results in the formation of distinct pockets on the receptor. One pocket  
10 which is distal to the coactivator binding site interacts with the peptide. As a result of this interaction, an additional conformational change occurs precluding the interaction between the coactivator and the receptor.

#### *Example 5*

15 Figure 20 shows a similarity analysis of the data pictured in Figure 7. Each ligand has a five element footprint, the elements corresponding to the normalized transcriptional response which it induced in a mammalian two-hybrid system presenting either the apo-receptor (control) or the receptor in  
20 the presence of one of the peptides  $\alpha\beta 1$ ,  $\alpha 2$ ,  $\alpha\beta 3$  or  $\alpha\beta 5$ .

*Example 101*

One of the distal steps in transcriptional activation by estrogen receptor (ER) is the recruitment by ligand-bound receptor of one of a number of coactivator proteins. This activity permits ER to interact with the general transcription machinery and exert its regulatory actions on target gene promoters. It has now emerged that one effect of agonist binding is to induce a conformational change within ER, permitting the interaction of ER helices 3 and 12, and the subsequent formation of a pocket which allows the coactivator proteins to dock. These observations suggest that receptor antagonists inhibit ER transcriptional activity by affecting the formation of the coactivator binding pocket and reducing the affinity of ER for coactivators. Although an ER-specific coactivator protein remains to be identified, several coactivators have been identified which potentiate the transcriptional activity of ER and other members of the steroid receptor superfamily. Furthermore, the finding that these coactivators use a highly conserved LXXLL motif to interact with the receptors made it uncertain as to whether receptor-cofactor interactions were determined by simple competition or if there was some specificity built into the system.

In order to address these possibilities, we undertook a molecular approach to dissect the LXXLL-ER interaction and to evaluate the role of flanking sequences in influencing these interactions. We utilized phage display technology to screen  $10 \times 10^7$  variations of the core LXXLL motif. Using estradiol-activated ER as a target, we identified a number of phage which encoded high affinity ER-interacting peptides. Using the sequence information derived from these phage, we constructed a series of GAL4-peptide fusions and assessed their ability to interact with ER $\alpha$ , ER $\beta$ , GR and PR using a two-hybrid assay in mammalian cells. The results of this assay confirmed that the LXXLL motif was permissive for nuclear receptor binding but it also revealed that sequences flanking this motif were important determinants of specificity. Thus, as expected, not all LXXLL



motifs are the same. This suggests that within a cell, specificity and not just mass action influences the ability of a nuclear receptor to find a required cofactor. In an effort to understand the mechanism underlying this observed  
5 specificity, we assayed the ability of these peptide fusions to interact with a series of ER helix-12 mutants. Using this approach we noticed that mutation of the conserved hydrophobic residues in this helix abolished ER-AF-2 function and blocked the interaction of all LXXLL peptides with ER. Disruption of  
10 helix 12 by mutating the three conserved charged residues (D538N/E542Q/D545N) prevented most peptides from binding and also abolished AF-2 function. However, a large number of the LXXLL-containing peptides studied were unaffected by this manipulation. This is an important observation since the  
15 latter mutation also blocks the interaction of ER with GRIP-1 and SRC-1. Cumulatively, our data indicate that the steroid receptors display distinct preferences for different classes of LXXLL motifs, suggesting a molecular basis for cofactor-receptor specificity. Importantly, however, they also indicate  
20 that Af-2 function and coactivator binding are not synonymous, a result which indicates that there are likely to be additional cofactors distinct from SRC-1 and GRIP-1 which remain to be discovered.

Plasmids: All the Gal4DBD-peptide fusions were  
25 constructed as follows: DNA sequences code for the peptides were excised from mBAX vector with XhoI and XbaI restriction enzymes and subcloned into pMsx vector derived from pM vector (Clontech) with a linker sequence to generate infram SalI and NheI sites for cloning. VP16ER-a construct was generated by  
30 polymerase chain reaction (PCR) of full length human ER- $\alpha$  cDNA with primers containing EcoRI flanking both 5' and 3' ends. The PCR product was then subcloned into pVP16 vector (Clontech) to generate the VP16-ER $\alpha$  fusion with VP16 located at the N-terminus of ER $\alpha$  cDNA, pVP16ER- $\beta$ , pVP16-RAR $\alpha$ , and pVP16-RXR $\alpha$   
35 were generated in a similar fashion. pVP16VDR is a generous gift from J. W. Pike (University of Cincinnati, Cincinnati, OH); VP16TR $\beta$  expression plasmid (pCMX-VP-F-hTR $\beta$  was provided by D.D. More (Baylor College of Medicine, Houston, TX); VP16Gr,

VP16PR-a, VP16PR-b, and VP16AR were gifts from J. Miner (GR), D.X. Wen (PR-a and PR-b), and K. Marschke (AR) (Ligand Pharmaceuticals, San Diego, CA). VP16-ER mutant constructs were generated by excision of mutant ER cDNAs from ER expression plasmids (ER-TAF1, ER-LL and ER-535 stop plasmids, Tzukerman et al. Mol. Endocrinol. 1994(8):21-30 and Norris et al., J. Biol. Chem. (273):6679-6688, 1998) and subcloned into pVP16 vector. Mammalian expression plasmids for ER $\alpha$ , ER $\beta$ , and ER179C, as well as 3xERELuc receptor construct were described elsewhere (Tzukerman et al. Mol. Endocrinol. 1994 (8):21-30). 5xGal4Luc3 construct was modified from 5xGal4-TATALuc plasmid (a gift from X.F. Wang, Duke University, Durham, NC) where the luciferase gene was replaced by a modified version of luciferase cDNA from pGL3 basic vector (Promega), GRIP-1 and SRC-1 constructs were generated by subcloning PCR products corresponding to GRIP-1 a.a. 629-760 and SRC-1 a.a. 621-765 into pM vector (P.H. Giangrande, unpublished). All PCR products were sequenced to ensure the fidelity of the resultant constructs.

#### 20 Example 101.1

A focused random peptide library (X<sub>7</sub>-LXXLL-X, X=any AA, L=Leu) was constructed and displayed on M13 phage.

Baculovirus expressed full length ER- $\alpha$  was treated with 10<sup>-6</sup> M of 17 $\beta$  estradiol and immobilized on 96-well Immulon-4 plates as selection targets. M13 phage-based random peptide libraries were incubated with target proteins in the wells, ER binding phage were retained while the unbound phage were washed away. Bound phage were eluted by low pH buffer, amplified in DH5 $\alpha$ F' cells and subjected to subsequent round of selection. The selection processes were repeated 2-3 times to enrich for EB bonding phage. Individual phage were plaque purified, amplified and their binding characteristics were examined by ELISA. Phage that bound to ER only in the presence of estradiol were selected and the peptide sequences were deduced by DNA sequencing (Table 101).

The LXXLL motif-containing peptides are major binding species in the affinity selection when estradiol activated ER $\alpha$  was used as a target. ER4 (Table 101) binds to agonist occupied ER but not partial agonist- or antagonist-occupied ER.

#### 5 Example 101.2

The ability of ER4 peptide to interact with ER- $\alpha$  in mammalian cells was assayed in a mammalian two-hybrid system. The ER4 peptide sequence was fused to Gal4 DNA binding domain (GAL4DBD) while the full length ER was fused to the VP16  
10 transactivation domain. The interaction between ER4 peptide and ER $\alpha$  is measured by the expression of 5xGal4Luc3 reporter gene. HepG2 cells were transiently transfected with (Fig. 14A) ER $\alpha$  expression vector and reporter 3xERELuc or (Fig. 14B) Gal4DBD-ER4, VP16-ER $\alpha$  and 5xGal4Luc3, and treated with  
15 different ER ligands. Luciferase activity was normalized to the activity of the cotransfected pCMV $\beta$ gal. The ability of ER4 to interact with ER- $\alpha$  in the presence of different ER-agonists paralleled that of ER transactivation function as assayed with 3xERELuc reporter. However, partial agonists or antagonist  
20 inhibited this interaction (Fig. 14C) and (Fig. 14D). Therefore, the LXXLL containing peptides provide a sensitive probe for AF2 activation.

#### Example 101.3

To test whether different LXXLL motif peptides interact  
25 within the same region of ER, mammalian two hybrid assays were used. Selected peptide sequences and different ER mutants (ER-LL, ER-3X, ER-535 STOP) were expressed as fusion proteins to Gal4DBD and VP16 (TAD), respectively. The binding affinity of different peptides (ER4, F6, D47, C33, D22, D48) to wild type  
30 ER and the three ER mutants were measured by the expression of 5xGal4Luc3 reporter construct. GRIP-1\* and SRC-1\* constructs contain the center 3 copies of LXXLL motif (a.a. 629-761 for GRIP-1 and a.a. 622-765 for SRC-1) fused to Gal4DBD. The results are shown in Figure 15.

## Example 101.4

HeLa cells were transfected with ER $\alpha$  expression plasmid (RST7ER $\alpha$ ), 3xERELuc reporter, pCMV $\beta$ gal along with different peptide-Gal4DBD fusion constructs. pM is the Gal4DBD control  
5 without peptide fusion. Luciferase activity was measured and normalized (Fig. 16). The ability of different peptides to disrupt ER $\alpha$  transcriptional activity correlates with the affinity of these peptides to ER $\alpha$  as measured in mammalian two-hybrid assays in [Figure 15(A)]. One exception is that the  
10 GRIP-1 construct although showed relatively weaker binding affinity to ER in mammalian two-hybrid assays, demonstrated to be an excellent candidate to disrupt ER transcriptional activity. Two copies of LXXLL motifs interact synergistically to disrupt ER transcriptional activity. 2XF6: two copies of  
15 F6 peptide was constructed in tandem with a 54-amino acid spacing linker that has the same sequence as in that in between GRIP-1 NR box II and NR box III. F6G:Gal4DBD-F6 fusion with only one copy of F6 peptide plus the linker. Transient transfection was performed in HeLa cells with 3xERELuc,  
20 RST7ER $\alpha$ , pCMV $\beta$ gal and increasing amount of Gal4DBD-peptide fusion constructs as indicated in the X-axis.

## Example 101.5

LXXLL containing peptides disrupted AF2 function in HepG2 cells, but did not totally abolish wtER transactivation  
25 function in HepG2 cells, where the AF-1 function is dominant (Fig. 17). However, in the same context, the transcriptional activity of a truncated form of ER (ER179C) that lacks the AF-1 domain was diminished by LXXLL containing peptides. HepG2 was transfected with either wtER or ER179C expression plasmids  
30 along with 3xERELuc reporter, Gal4DBD-peptide fusion constructs, and pCMV $\beta$ gal to normalize for transfection efficiency. After transfection, cells were induced with different concentrations of 17 $\beta$ -estradiol for 16 h before assaying.

## 35 Example 101.6

The interactions between different LXXLL motifs and different nuclear receptors were assayed in a mammalian two-hybrid system (Fig. 18). Full length receptors and selected peptides were expressed as VP16 and Gal4 DBD fusions, respectively. The strength of the interactions was measured by the activity of the 5xGal4Luc3 reporter gene. NH: no hormone; H: hormone treatments. Hormones used in this experiment:  $10^{-7}$  M  $17\beta$  estradiol for ER- $\alpha$  and ER- $\beta$ ,  $10^{-7}$  M progesterone for PR-a and PR-b,  $10^{-7}$  M dexamethasone for GR,  $10^{-7}$  M 9-cis retinoic acid for RAR and RXR,  $10^{-7}$  M T3 for TR,  $10^{-7}$  M 1,25-dihydroxy Vit.D3 for VDR, and  $10^{-6}$  M  $5\alpha$ -dihydrotestosterone for AR.

#### Example 101.7

Peptide #293 (ER beta 15e2, sequence SSIKDFPNLISLLSR) was affinity selected from phage display of estradiol activated ER $\beta$ . It contains the LXXLL motif. It showed selective interactions with ER $\beta$ , TR $\beta$  and RAR $\alpha$  but not with other receptors tested as shown in Figure 7. Expression of this peptide did not interfere with the transcriptional activity of ER $\alpha$  but strongly disrupted the transcriptional activation by ER $\beta$  (Fig. 19). HeLa cells were transfected with either ER $\alpha$  or ER $\beta$  expression plasmids along with 3xERELuc reporter, pCMV $\beta$ gal and peptide-DBD fusion constructs as indicated. Cells were treated with different concentrations of  $17\beta$ -estradiol for 16h before assaying.

#### Conclusions:

● Peptides with LXXLL motifs have related but different activities. Flanking sequences determine:

1. their affinity for nuclear receptors.
2. the requirements for a functional AF2 in ER- $\alpha$  for interaction.
3. their specificity of interaction with different nuclear receptors.

● LXXLL motifs can knock out the estradiol induced ER- $\alpha$  transcriptional function in HeLa cells where both AF1 and AF2 functions are required for activation. However, in the cell context where AF1 function is dominant (such as HepG2 cells),  
5 LXXLL motifs cannot totally abolish the estradiol activated transcriptional activity. This observation implies two possible explanations:

1. In HepG2 cells, the AF1 activity is due to a different coactivator that contacts primarily the AF1  
10 region. Therefore, disruption of the interaction between ER- $\alpha$  and LXXLL-containing cofactors does not disrupt AF1 function.

2. A HepG2 specific cofactor contacts both AF1 and AF2 domains. Disruption of the AF2 binding site is not  
15 sufficient to knock out the interaction of cofactor-ER interaction.

● Peptides with Estrogen Receptor specific LXXLL-containing motifs can be obtained by phage display screening and, if active and pharmaceutically acceptable in humans, be  
20 used as receptor-specific antagonists.

*Example 201 Application of the Technology to G-Protein-Coupled Receptors and G  $\alpha$  Subunits*

The in vitro drug identification system described above can also be extended to other classes of biological signal  
5 modulating proteins such as the serpentine receptors (also known as the G protein coupled receptors and seven transmembrane spanning receptors) and their cognate G proteins. We will refer to these as GPCRs. GPCRs have been extensively exploited as targets for drug discovery in many therapeutic  
10 areas such as gastrointestinal, cardiovascular and neurological diseases. The ability to rapidly and inexpensively identify drugs that activate or block GPCRs would be of great utility to the pharmaceutical industry.

All GPCRs have at least two functional domains. One is  
15 the ligand binding domain on the external surface and the other is the G protein binding domain that is on the intracellular surface.

In their quiescent state the G proteins that are activated by GPCRs exist as G protein ( $\alpha\beta\gamma$ ) heterotrimers containing  
20 guanine diphosphate (GDP) bound to G $\alpha$  subunits. GPCRs activate their cognate G proteins by acting as guanine nucleotide exchange factors (GEFs). Upon GPCR activation, free GTP replaces GDP bound to the  $\alpha$  subunit of the G protein. The GTP-bound G $\alpha$  subunit and G $\beta\gamma$  then disassociate and regulate the  
25 function of second messenger enzymes and ion channels. GPCRs activate their cognate G proteins by acting as guanine nucleotide exchange factors (GEFs). Before GPCRs can activate G proteins, they must be switched from an inactive to an active state by the action of the appropriate ligand. GPCRs have  
30 little or no detectable affinity for their cognate G proteins until activated. Chemicals that mimic the action of GPCR ligands are known as agonists and induce a change in the GPCR such that it acquires selective affinity for its cognate G protein. Chemicals that block the action of the GPCR ligands  
35 are known as antagonists and prevent the induction of structural changes necessary for the GPCR to bind to the

cognate G protein.

BioKeys (peptides and similar molecules) that probe GPCRs can be derived from both the GPCRs themselves or their cognate  $G\alpha$  subunits. The  $G\alpha$  subunits can indicate GPCR activation because when GPCRs are activated GTP is bound to the  $G\alpha$  subunit, and when they are inactive GDP is bound. For GPCRs these BioKeys will specifically recognize two basic functional domains, the ligand binding site and the activated receptor via the G protein-binding site. In the case of  $G\alpha$  subunits the BioKeys will specifically recognize the GDP or GTP bound forms. Thus, four classes of BioKeys can be identified, two each for  $G\alpha$  subunits and GPCRs. Such Biokeys can be of immense value for the identification of new therapeutic agents (drugs) using in vitro screening methods.

At present, drugs that act on GPCRs are generally identified in either of two ways. One (the cell based assay) is the use of whole cell assays that are very cumbersome and expense to carry out. The other (ligand displacement assay) is the use of labeled ligands to determine the ability of a test substance to compete for the binding of the ligand to the GPCR. While the latter is substantially less expensive and more convenient to carry out, it is not possible to distinguish agonists from antagonists and thus the usefulness of such an assay is limited.

Through the use of BioKeys specific to each of the basic functional domain of GPCRs, we can carry out simple, inexpensive in vitro screens for both agonists and antagonists to GPCRs and can distinguish the complete range of activities for such compounds from pure agonists, to partial agonists to complete antagonists.

Example A: Screen for agonists and antagonists to the beta-two adrenergic receptor (AR). AR is activated by ligands such as epinephrine and isoproterenol. These ligands are agonists and are useful for the treatment of diseases such as asthma and severe allergic reactions. Antagonists to AR are useful for regulating cardiac function and the treatment of hypertension and cardiac arrhythmias.



Class I BioKeys to AR are identified by affinity selection of high affinity (better than 50 micromolar) peptides from BioKey libraries. AR can be produced using recombinant techniques known to those in the art using systems such as the  
5 baculovirus expression system in insect cells. AR containing membranes or purified AR can be used for the affinity selection of BioKeys to the ligand binding site. Selectivity for that site can be confirmed by the displacement of the BioKey by the agonist isoproterenol.

10 In a similar manner, Class II BioKeys can be identified to the G protein binding domain of activated AR. First, AR is pretreated with an excess of an agonist e.g. isoproterenol to induce the AR into an "active" conformation. BioKeys are selected as described herein and selectivity and specificity  
15 is confirmed by their ability to bind to agonist treated but not to untreated AR or antagonist treated AR.

Class III BioKeys can be identified to the GDP-bound form of  $G_{\alpha}$  subunits. Purified  $G_{\alpha}$  subunits are produced and purified using recombinant techniques and expression in  
20 bacterial cells. Purified  $G_{\alpha}$  subunits are pretreated with an excess of GDP to induce the "inactive" conformation or GDP-bound form of  $G_{\alpha}$  (GDP- $G_{\alpha}$ ). BioKeys are selected as described herein and selectivity and specificity is confirmed by their ability to bind to GDP treated, but not to GTP treated  $G_{\alpha}$   
25 subunits.

In a similar manner, Class IV BioKeys can be identified to the GTP-bound form of  $G_{\alpha}$  subunits. Purified  $G_{\alpha}$  subunits are pretreated with an excess of GTP to induce the "active" conformation or GTP-bound form of  $G_{\alpha}$  (GTP- $G_{\alpha}$ ). BioKeys are  
30 selected as described herein and selectivity and specificity is confirmed by their ability to bind to GTP treated, but not to GDP treated  $G_{\alpha}$  subunits.

Representative BioKeys to all four classes can be labeled with a suitable moiety as described elsewhere herein such as  
35 europium labeled streptavidin and used in a drug screen using fluorescence measuring devices. Many other means of using BioKeys as surrogate ligands will be apparent to those skilled

in the art of drug compound screening.

As can be seen from Table 5, it is very easy to distinguish three classes of compounds from a chemical compound collection. Inactive compounds do not bind to relevant functional domains on the AR and thus no change in signals from either BioKey is seen. For GPCRs, agonists are easily identified due to their ability to induce the AR into a conformational state such that the G protein binding domain is capable of binding class II BioKeys that are surrogates for the AR's cognate G protein. Agonists that bind to the ligand binding site will also lead to a measurable decrease in class I BioKey binding. Antagonists are identified by their ability to bind to the ligand binding domain and hence are capable of blocking the natural ligand from binding. However, unlike agonists, they have no ability to induce the activation of the receptor; hence there is no change in the conformation of the AR's G protein binding domain and thus no change in ability to bind class II BioKeys.

Alternatively, screening for compound agonists or antagonists can be performed using AR-containing membranes, Gs and BioKeys specific for GTP-Gs $\alpha$  or GDP-Gs $\alpha$ . Agonists will activate the receptor and result in the formation of GTP-Gs $\alpha$ . The signal from BioKeys specific to GDP-Gs $\alpha$  will decrease if an agonist is binding the receptor. Likewise, the signal from BioKeys specific to GTP-Gs $\alpha$  will increase if an agonist is bound to AR. Antagonists are not capable of activating the receptor and therefore are not able to activate G proteins. The G protein will then remain a heterotrimer containing GDP bound to its Gs $\alpha$  subunit. Screen for antagonist using AR-containing membranes pretreated with an agonist. The signal from BioKeys specific to GTP-Gs $\alpha$  will decrease if an antagonist is binding the receptor. Likewise, the signal from BioKeys specific to GDP-Gs $\alpha$  will increase if an antagonist is bound to AR.

This system can be readily extended to other GPCRs for which one has access to a natural ligand or an agonist.

#### Example 401 Fingerprinting of Modulators of the Glucocorticoid

**Receptor**Peptide Sequences

F6: GHEPLTLLERLLMDDKQAV

 $\alpha/\beta$ III: SSWDMHQFFWEGVSR5  $\alpha/\beta$ V: SSPGSREWFKDMLSR $\alpha$ II: SSLTSRDFGSWYASR

Note that these peptides, while originally identified as peptides which bind the estrogen receptor, are usable in fingerprinting of modulators of the glucocorticoid receptor.

10 The ER binding peptides work on the glucocorticoid receptor because nuclear receptors have structural similarities. The exact nature of these similarities are not known although there are sequence similarities. Identical coactivator proteins bind both receptor and contain LXXLL motifs. Thus it is not

15 surprising that our LXXLL peptides might also bind both receptors in the presence of agonist. See McInerney, et al., Genes & Development, 12:3357-68 (1998); Nolte, et al., Nature, 395:134-143 (September 10, 1998).

20 Titration of GR vs. F6 with Deoxycorticosterone and Dexamethasone

Yeast strain EGY48 (MAT $\alpha$  trp1 his3 ura 3 leu2::6LexAop-Leu2) was transformed with plasmids pJK103 (2 $\mu$ M, 2LexAop-LacZ), pJG4-5-F6 (2 $\mu$ M, LexADBD-F6 peptide), and pEG202-GR (2  $\mu$ M, B42AD-Glucocorticoid Receptor  $\alpha$ ). The resulting transformed

25 strain was grown overnight in media containing galactose as the sole carbon source to induce expression of GR. Deoxycorticosterone and dexamethasone were serially diluted into 100  $\mu$ l of media in a 96-well microplate. 100  $\mu$ l aliquots of the overnight yeast culture were added to the microplate

30 wells and incubated at 30°C for 3 hours. To monitor the interaction of the F6 peptide with GR, a kinetic assay for  $\beta$ -galactosidase activity was performed. The cell density in each was determined by reading the OD<sub>650</sub>. Yeast were pelleted by centrifugation for 5 minutes at 3000 rpm and the media removed.

35 20  $\mu$ l of 1xZ buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl 1 mM MgSO<sub>4</sub>, 7 mM 2-mercaptoethanol) containing 2.5% CHAPS detergent was added and briefly mixed by agitation. Following a 5

minute incubation at room temperature, 100  $\mu$ l of 1xZ buffer containing 40  $\mu$ g o-Nitrophenyl  $\beta$ -D-Galactopyranoside (ONPG) was added to each well. Color development was monitored by measuring the change in OD<sub>405</sub> referenced to OD<sub>650</sub> over 10 minutes (20 second intervals).  $\beta$ -galactodase activity is expressed as  $\Delta(OD_{405}-OD_{650})/\text{initial } OD_{650}$ .

#### Interaction of GR with peptides

Yeast strain EGY48 (MAT $\alpha$  trp1 his 3 ura3 leu2::6LexAop-Leu2) was transformed with plasmids pJK103 (2  $\mu$ M, 2LexAop-LacZ), pEG202-GR(2  $\mu$ M, LexADBBD-F6, - $\alpha$ / $\beta$ III, - $\alpha$ / $\beta$ V, or - $\alpha$ Ii peptides). The resulting transformed strain was grown overnight in media containing galactose as the sole carbon source to induce expression of GR. The culture was diluted to an OD<sub>600</sub> of 0.1 in 10 ml of media and deoxycorticosterone, dexamethasone, corticosterone, or  $\beta$ -estradiol were added to a final concentration of 1  $\mu$ M. The cultures were incubated at 30°C for 3 hours. Preparations of protein were made by lysing the yeast by agitation with glass beads. The cellular debris was removed and the protein concentrated by precipitation with 50% ammonium sulfate for 30 minutes at 4°C. The protein pellet was suspended in storage buffer (100 mM HEPES 50 mM EDTA 40% glycerol 7 mM 2-mercaptoethanol, pH8) and protein concentrations determined. To determine the interaction of the peptides with GR, an end point assay for  $\beta$ -galactosidase activity was performed. 10  $\mu$ g of protein extract was diluted into a final volume of 100  $\mu$ l 1xZ buffer and color development was initiated by the addition of 80  $\mu$ g of ONPG. The reactions were stopped by addition of 30  $\mu$ l of 1M Na<sub>2</sub>CO<sub>3</sub> and the time of development noted.  $\beta$ -galactosidase activity is expressed as 1000\*OD<sub>405</sub>/min/mg protein.

*All references cited anywhere in this specification are hereby incorporated by reference.*

Table A: List of Proteins for Fingerprinting Analysis:

	Receptors	Modulators of Activity
	Nuclear receptors	
5	Estrogen Receptor $\alpha$	Estradiol (agon),
	and $\beta$	tamoxifen (antag), ICI
		182,780 (antag), Raloxifene,
		(antag),
	Progesterone	Progestins, estrogens
		(agon), RU486 (antag),
		ZX98299, (antag), onapristone
		(antag)
	Androgen	Dihydroxytestosterone
		(agon), hydroxyflutamide
		(antag)
	Glucocorticoid	Cortisone (agon),
		dexamethasone (agon)
10	mineralocorticoid	Aldosterone (agon),
		spironolactone (antag)
	Retinoic acid	9-cis retinoic acid
		(agon)
	Thyroid	Thyroid hormone (agon)
	Vitamin D3	Vitamin D3 (agon)
	PPAR(s)	Eicosinoids (agon),
		oxidized LDL (agon)
15	LXR	Oxidized cholesterol
		metabolites (agon)
	FXR	Farnesoid metabolites
		(agon)
	BXR	3-aminoethyl benzoate
		(agon)
	SXR	Steroids (agon),
		phytoestrogens (agon),
		xenobiotics (agon)
	Orphan Nuclear Receptors	
20	Nurr1	
	Nor1	

	NGF1-B	
	ERR1	
	SHP	
	HNF-4	
5	Coup-TF II	
	Tyrosine Kinase Receptors	
	Epidermal growth factor	EGF (agon), ATP
	Insulin	Insulin(agon), ATP
10	Platelet derived growth factor	PDGF(agon), ATP
	G-Protein Coupled Receptors	
15	$\beta$ -adrenergic receptor	Isopreterenol (agon), alprenolol (antag)
	Rhodopsin	
	Dopamine D2	Dopamine (agon), haloperidol (antag)
	opiod	Leu-enkephalin (agon), Naltrindole (antag)
20	Endothelin	Endothelin 1(agon), BQ-123 (antag)
	Erythropoietin receptor	Erythropoietin
	FAS ligand receptor	FAS ligand
	Interleukin receptor	Interferon (agon) IL-6 (agon)
	Signal Transduction	
25	Proteins	
	Kinases	
	Protein Kinases	
	Protein kinase C	diacylglycerol (agon), staurosporine (antag)
	Tyrosine kinase	ATP, genistein (antag)

	Serine kinase	ATP
	Threonine kinase	ATP
	Nucleotide kinase	ATP
	Polynucleotide kinase	ATP, DNA, PO <sub>4</sub>
5	Phosphatase	
	Protein Phosphatase	
	Serine/threonine	
	Tyrosine	
	Nucleotide phosphatase	
10	Acid phosphatase	
	Alkaline phosphatase	
	pyrophosphatase	
	Cell Cycle Regulators	
	Cyclin CDK-2	
15	CDC2	
	CDC25	
	p53	
	Retinoblastoma	
	GTPases	
20	Large G proteins	
	Gαs	suramin (antag), mastoparin (agon)
	Small G Proteins	GAPs (ag), GEF (antag)
	Rac	
25	Rho	
	Rab	
	Ras	
	Proteases	
	Endoprotease	
30	Exprotease	
	Metalloprotease	
	Serine protease	
	Cysteine protease	
	Nucleases	
35	Polymerases	

Ion Channels

Chaperonins

Heat shock Proteins

Viral Proteins

5 Deaminases

Nucleases

Deoxyribonuclease

Ribonuclease

Endonucleases

10 Exonucleases

Polymerases

DNA dependent RNA polymerase

DNA dependent DNA polymerase

Telomerase

15 Primase

Helicase

Dehydrogenase

Aminoacyl tRNA synthetases

Transferases

20 Peptidyl transferase

Transaminase

Glycosyltransferase

Ribosyltransferase

Acetyl transferases

25 Acyltransferases

Hydrolases



## . Carboxylases

## Isomerases

Dismutase

Rotase

5 Topoisomerase

## Glycosidase

Endoglycosidase

Exoglycosidase

## Deaminase

10 Lipases

## Esterases

Sulfatases

## Cellulase

## Lyases

15 Reductases

## Synthetase

DNA binding proteins

RNA binding proteins

Nuclear receptor coactivators

20 Ligases

RNA

DNA

Tumor suppressor

Adhesion molecule

Oxygenase

Peroxidase

Transporters

5            Electron transporters

            Protein transporters

            Peptide transport

            Hormone transport

                 Serotonin

10                   DOPA

            Nucleic acid transport

Transcription factors

Neurotransmitters

Information carrier/storage

15            Antigen recognition protein

            MHC I complex

            MHC II complex

            Antag=antagonist of receptor

            agon=agonist of receptor

Table B: Target Tissues  
Circulatory and Lymphatic Systems

	Heart
	Walls
5	Valves
	Blood Vessels
	Blood Cells
	Erythrocytes
	Platelets
10	Leukocytes
	Lymph Nodes
	Lymphatic Vessels
	Spleen
	Thymus
15	Tonsils

Respiratory System

	Lungs
	Trachea
	Bronchi
20	Bronchioles
	Alveoli
	Pleura
	Pharynx
	Larynx
25	Trachea

Endocrine System

	Pituitary Gland
	Thyroid Gland
	Parathyroid Gland
30	Adrenal Gland
	Adrenal Medulla
	Adrenal Cortex
	Pancreas
	Islets of Langerhans
35	Liver
	Gall Bladder

	Mammary Glands
	Central Nervous System
	Brain
	Neurons
5	Glial Cells
	Spinal Cord
	Nerves
	Peripheral Nervous System
	Eye
10	Retina
	Lens
	Ear
	Eardrum
	Ampullae
15	Spiral organ of Corti
	Nose
	Olfactory bulbs
	Tongue
	taste buds
20	Digestive System
	Tongue
	Salivary Gland
	Pharynx
	Esophagus
25	Stomach
	Small Intestine
	Large Intestine
	Urinary System
	Kidney
30	nephrons
	Bladder
	Male Reproductive System
	testes
	prostate gland

bulbourethral (Cowper's) glands  
penis  
sperm cells

Musculoskeletal System

5           bones (various)  
            bone marrow  
            joints (various)  
            muscles (various)  
            ligaments (various)

10          Female Reproductive System

            Ovaries  
            Uterus  
            Bartholin's Glands  
            Paraurethral Glands  
15          Egg Cells

Integumentary System

            Skin  
                epidermis  
                dermis  
20          hypodermis  
            sweat glands  
            sebaceous glands  
            hair  
            nails

Table 1

Peptides the Bind to the Unliganded (unactivated) Estrogen Receptor		
	Sequence	Phage #
5	S R W E S P L G T W E W S R	4
	S A A P R T I S H Y L M G G	48
	S S W V R L S D F P W G V S R	1
	S S W D R L S D F P W G V S R	2
	S S W I R L R D L P W G E S R	3
10	S S W V L L R D L P W G S R	31
	S S W V V L R D L P W G S R	29
	S S C K W Y E K C S G L W S R	7
	S S G I C F F W D G C F E S R	35
	S R N L C F F W D D E Y C S R	41
15	H H H R H P A H P H T Y G G	47

Table 2

Peptides that Bind to the Estradiol Activated Receptor		
	Sequence	Phage #
20	S R A G L L S D L L E G K S R	1/2
	S S R S L L R D L L M V D S R	6
	S S N K L L Y N L L K M E S R	22
	S S K S L L L N L L S T P S R	23
	H S F P R E S L L V R L L Q G G	42
25	S R L E M L L R S E T D F S R	3
	S R L E E L L K W G S V T S R	11
	S R L E Q L L K E E F S Y S R	21
	S R L E Q L L R S E P D F S R	27
	S R L E D L L R A P F T T S R	28
30	S R L E S L L R F G Q L D S R	29
	S S R L L S L L V G D F N S R	19/20
	S R L E E L L L G T N R D S R	30
	S R L K E L L L L P T D L S R	15
	S R L E C L L E G R L N C S R	34
35	S S K L Y C L L D E S Y C S R	35
	S R L S C L L M G F E D C S R	36
	S S K L I R L L T S D E E L S R	37
	S S R L M E L L Q E G Q G W S R	40
	S S N H Q S S R L I E L L S R	4
40	S S R L W Q L L A S T D T S R	16
	S S N S M L W K L L A A P S R	13/14
	S S K T L W R L L E G E R S R	17
	S R A G P V L W G L L S E S R	32
	S S L T S R D F G S W Y A S R	5
45	S S W V R L S D F P W G V S R	24/25
	S S E Y C F Y D S A H C S R	33
	S R S L L E C H L M G N C S R	7
	S S E L L R W H L T R D T S R	8
	S R L E Y W L K W E P G P S R	12
50	S R S D S I L W R M L S E S R	31
	S S K G V L W R M L A E P V S R	38/39
	H S H G P L T L N L L R S S G G	41
	S S A G G G A P A G S T P S R	26

Other ER binding peptides include

SSKYSYSRSSEGHSR

SSYQWETHSDKWRSR

SSVTKKALTIKDSR

- 5 The latter two are weak binders of ER in presence of estradiol.

Table 3: Phage/Peptide Classification

	<u>Class 1</u>	<u># and isolation method</u>
5	S S N H Q S S R L I E L L S R	#4 ER + estradiol
	S R L K E L L L L P T D L S R	#15 ER + estradiol
	S S K L Y C L L D E S Y C S R	#35 ER + estradiol
	H G P L T L N L L R S S G G	#41 ER + estradiol
	S R L E Y W L K W E P G P S R	#12 ER + estradiol
	<u>Class 2</u>	
10	S S C K W Y E K C S G L W S R	#7 ER
	S S E Y C F Y W D S A H C S R	#33 ER + estradiol
	S S W V L L R D L P W G S R	#31 ER
	S S W V R L S D F P W G V S R	#24 ER + estradiol
	<u>Class 3</u>	
15	S S L T S R D F G S W Y A S R	#5 ER + estradiol
	<u>Class 4</u>	
	S R T W E S P L G T W E W S R	#13 ER
	<u>Class 5</u>	
	S A A C A T I S H Y L M G G	#48 ER



Table 4		Characteristics of the 5 Phage Classes			Competition with LXXLL peptide
		Affinity for unliganded ER $\alpha$	Affinity for unliganded ER $\beta$	Effect of Agonist (Estradiol)	
5	Class 1	+	+++	$\uparrow$ binding to $\alpha$ & $\beta$	+ $\alpha$
	Class 2	+++	++	No effect	+ $\beta$
	Class 3	++	+	$\uparrow$ binding to $\alpha$ no effect on $\beta$	- $\alpha$
	Class 4	+++	++	binding to $\alpha$ no effect on $\beta$	- $\beta$
	Class 5	++(+)	+++	binding to $\alpha$ & $\beta$	+ $\alpha$
10					- $\beta$

Table 5

	Agonist compound	Antagonist compound	Inactive compound
* BioKey I	No change or decreased signal	Decreased signal	No change
BioKey	Increased signal	No change or decreased signal	No change
5 II @			

\* BioKey specific to the ligand binding site

@ BioKey specific to the G protein binding site on the activated receptor.

Table 6A: Fingerprint Analysis for Agonists and SERMs on ER  $\alpha$ , by Peptide Class

Class	Estradiol	Estradiol	estriol	Tamoxifen	4-OH	Nafoxidine	Clomiphene
1	+++	+++	+++	+	+	++	+
2	+/-	+/-	+/-	+	+	+	+
3	++	++	++	++	++	++	+++
4	--	--	--	+	+	+	+
5	-	-	-	+	+	+	+

Table 6B: Fingerprint Analysis for Agonists and SERMs on ER  $\beta$ , by Peptide Class

Class	Estradiol	estriol	Tamoxifen 4-OH	Nafoxidine	Clomiphene
1	++	++	-	+/-	+/-
2	+/-	+/-	+	++	++
3	+/-	+/-	+	++	++
4	+/-	+/-	++	++	++
5	-	-	+/-	+/-	-

Table 7: New  $\text{E}\alpha$  Peptide Sequences Immobilized on Plastic

Peptide name	Peptide Sequence	Isolated in the presence of receptor form	SERM present when peptide was identified
1PT	SRNLCFFWDDEYCSR	$\alpha$	Tamoxifen & ICI 182,780
2PT	SWDMHQFFWEGVSR	$\alpha$	Tamoxifen
3PT	SRWHGTLFWQDEQSR	$\alpha$	Tamoxifen
4PT	SSCKWYEKCSGLWSR	$\alpha$	Tamoxifen & ICI 182,780
5PT	SSRMGHVWYDWTFSR	$\alpha$	Tamoxifen
6PT	SSRLLGDFGGSVVS	$\alpha$	Tamoxifen
7PT	SSKYVFGFQVAGGSR	$\alpha$	Tamoxifen
8PT	SSWAGIKFGKPPHSR	$\alpha$	Tamoxifen
9PT	SSWSYSGKPTFLSSR	$\alpha$	Tamoxifen
10PT	SRDTGDMWGRGCSR	$\alpha$	Tamoxifen
11PT	SSGRYDPFVLNAASR	$\alpha$	Tamoxifen
12PT	SSSPWWSFNLRDMSR	$\alpha$	Tamoxifen
13PT	SSWPYLPKREEWASR	$\alpha$	Tamoxifen
14PT	SSGWIEQKLRGSFSR	$\alpha$	Tamoxifen
15PT	SSSATSIVQYQISR	$\alpha$	Tamoxifen
16PT	SSYLTGKSMMAISR	$\alpha$	Tamoxifen
17PT	SSWHSRWDLALGFSR	$\alpha$	Tamoxifen
18PT	SSGYWGGWDYGAGSR	$\alpha$	Tamoxifen
19PT	SRDNCGAGLWAGCSR	$\alpha$	Tamoxifen
1PI	SSSTPGWWEWDWASR	$\alpha$	Tamoxifen
2PI	SSYWDGSWRRKETCVSCSR	$\alpha$	ICI 182,780
3PI	SSRTAEDYCFADDDYWC	$\alpha$	ICI 182,780
4PI	SSRALALFPVGMESR	$\alpha$	ICI 182,780
5PI	SSDCESLTSYPHLKALCSR	$\alpha$	ICI 182,780
6PI	SSSTATALRDRLAYSR	$\alpha$	ICI 182,780
7PI	SSGKTRHYREGTSR	$\alpha$	ICI 182,780

Table 8: New ER $\alpha$ -ERE Peptide Sequence Information

Peptide name	Peptide Sequence	Isolated in the presence of receptor form	SERM present when peptide was identified
5			
E1-1	HSHNHHSPWLFRLGG	$\alpha$	Estradiol
E1-3	HSHPHHSHLLYKLMGG	$\alpha$	Estradiol
E1-4	HSHPLPPLLSRLLTGG	$\alpha$	Estradiol
E1-7	SRLTCLLQSNQWDSEQCSR	$\alpha$	Estradiol
I4-10	SSLTSRDFGSWYASR	$\alpha$	ICI
T3-1	SRTLQLDWTLYSR	$\alpha$	Tamoxifen
T1-10	SRLPPSVFSGSEVCLSR	$\alpha$	Tamoxifen
T2-10	SRFEIWKPEPGCVSSLENWE PGKRVCSR	$\alpha$	Tamoxifen
10			
T3-11	SRVFGVSGEVVLINGSSR	$\alpha$	Tamoxifen
1R	SRLCFGDWCMLGVDVLSR	$\alpha$	Raloxifen
2R	SSLNMVVDTPWCGKWCSR	$\alpha$	Raloxifen
3B	SSRPDAAFFGAKLSR	$\alpha$	Buffer
4B	SSRPSPSFWEKQLSR	$\alpha$	Buffer
5B	SSRPTAEWFRENLSR	$\alpha$	Buffer
6B	SRWWDTSWWLEELSR	$\alpha$	Buffer
1B	SSRIADLFWRLPSR	$\alpha$	Buffer
7B	SRSYHGEWGVWTLR	$\alpha$	Buffer
10B	SSDWCFGWGWCASEAVSR	$\alpha$	Buffer
9B	SRNWDWAALLELLPYPHPSR	$\alpha$	Buffer
1E	SSLTSRDFGSWYASR	$\alpha$	Estradiol
2E	SRSPILTHLLSLGSR	$\alpha$	Estradiol
3E	SSTGILWKLLTAESR	$\alpha$	Estradiol
9E	SSHGILWRLLSEGR	$\alpha$	Estradiol
11E	SRSDSILWRMLSESR	$\alpha$	Estradiol
4E	SRLVALLKSPWSVSR	$\alpha$	Estradiol
5E	SRLEELLIMDFWRSR	$\alpha$	Estradiol
6E	SSKLWQLLSSPIDSR	$\alpha$	Estradiol
14E	SSKLYCLLDESYSR	$\alpha$	Estradiol
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25			
30			

7E	SRSLLMDMLMSDDYVTVSR	α	Estradiol
8E	SSRLLACELMYEDADVCSR	α	Estradiol
15E	HSHSPLLMALLAPPGG	α	Estradiol
10E	SRLEYLRLGTYESR	α	Estradiol
13E	SSCLREILLYGACSR	α	Estradiol
16E	SSRTAEDYCFADDYWC	α	Estradiol
17E	SSLRCYLSSSKVDQWACSR	α	Estradiol
18E	SSYKPHSLLEWHLLGCTSR	α	Estradiol

Table 9: New ER $\beta$ -ERE Peptide Sequence Information

Peptide name	Peptide Sequence	Isolated in the presence of receptor form	SERM present when peptide was identified
1B- $\beta$	SRLHCLLDSSYCSSR	$\beta$	Buffer
2B- $\beta$	SRLHCLLDSSYCSSR	$\beta$	Buffer
3B- $\beta$	SSWPNTTFWERQLSR	$\beta$	Buffer
4B- $\beta$	SYSKEWFEERLNSR	$\beta$	Buffer
5B- $\beta$	SSMMREFFERELSR	$\beta$	Buffer
6B- $\beta$	SSGLPPNFERMLKSR	$\beta$	Buffer
7B- $\beta$	SSGPWLMHYLGGS	$\beta$	Buffer
8B- $\beta$	SSTSWLHHYLMGTSR	$\beta$	Buffer
9B- $\beta$	SRGGCECLGPWCLSR	$\beta$	Buffer
12B- $\beta$	SSEACVGRWMLCEQLGVSR	$\beta$	Buffer
14B- $\beta$	SSQVWPGPWRLVESR	$\beta$	Buffer
16B- $\beta$	SSSLGPWRLSELSR	$\beta$	Buffer
17B- $\beta$	SSSGPWRLWGLSIESR	$\beta$	Buffer
18B- $\beta$	SRECVGGWCLAEISR	$\beta$	Buffer
19B- $\beta$	SSIPPRSWWLSQLSR	$\beta$	Buffer
20B- $\beta$	SSWPGAEWFKQLSR	$\beta$	Buffer
21B- $\beta$	SSKLYCLLDESYCSR	$\beta$	Buffer
23B- $\beta$	HSYSSHPLLLSYLWGG	$\beta$	Buffer
24B- $\beta$	HSWLG PWRLSSIDLGG	$\beta$	Buffer



25B-β	HSTDMGWLRPWRLGG	β	Buffer
1T-β	SSVFTMDGKVALSR	β	Tamoxifen
2T-β	SRPYCLGDVWCLDSR	β	Tamoxifen
4T-β	SREWEDGFGGRWLSR	β	Tamoxifen
5T-β	SSWNSREFFLSQLSR	β	Tamoxifen
6T-β	SSTIMFDFFYERLSR	β	Tamoxifen
7T-β	SSARPWWLQFEGSSR	β	Tamoxifen
8T-β	SSQEEWLLPWRLASR	β	Tamoxifen
9T-β	SRLPPSVFSMCGSEVCLSR	β	Tamoxifen
10T-β	SSGPFYVGGMLWPADCLSR	β	Tamoxifen
12T-β	SREGWMGPWRLADSR	β	Tamoxifen
13T-β	SRNECIGPWCLTISR	β	Tamoxifen
14T-β	SSPGSREWFKDMLSR	β	Tamoxifen
15T-β	SSVASREWWVRELSR	β	Tamoxifen
16T-β	SRMFQVCGDEVCLSR	β	Tamoxifen
17T-β	SSDLHRDCLGVWCLSR	β	Tamoxifen
18T-β	SRLNGVFCHDSSDLWVCSR	β	Tamoxifen
20T-β	SRPGCLRGVWCLADTPPSR	β	Tamoxifen
21T-β	SSRLVPHSFWLDGLMHGSR	β	Tamoxifen
22T-β	SSISTYHMGWFYAMLSSR	β	Tamoxifen
23T-β	SSDLYSQMREFFQNLRSR	β	Tamoxifen
1E-β	SSRGLLWDLLTKDSR	β	Estradiol

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2E-β	SRHGILWDL LQGDSR	β	Estradiol
3E-β	SRLHDL LRLRDESPSR	β	Estradiol
4E-β	SRDWRSGFLYELL SR	β	Estradiol
5E-β	SSDTRSRLYELLSSSYTSR	β	Estradiol
6E-β	SRLEELLRVGVLT SR	β	Estradiol
7E-β	SRLEDLLRGDSK PQSR	β	Estradiol
8E-β	SSPTGHRLLLESLLNSNR	β	Estradiol
9E-β	SSILERLLGGGSAETV	β	Estradiol
10E-β	SRSPILWHLLQD GSR	β	Estradiol
11E-β	SSRTPILFSLLET SR	β	Estradiol
12E-β	SSIKDFPNLISLL SR	β	Estradiol
13E-β	SSGSSAGRLMMLLQDGVSR	β	Estradiol
14E-β	SREGLLMRLLIGDSR	β	Estradiol
15E-β	SSHCHTRLC SLLTSR	β	Estradiol
16E-β	SSRLCLLDAGQCSR	β	Estradiol
17E-β	SRNLLCLLDQEACSR	β	Estradiol
18E-β	SSLKCLLNSNFCSR	β	Estradiol
19E-β	SSLKCLLQSSPQKQPFCSR	β	Estradiol
20E-β	SSRTLLEHYLLG GSR	β	Estradiol
21E-β	SSAGLLEDMLR SRSR	β	Estradiol
22E-β	SSRCSSLLCEMLIQTKESR	β	Estradiol
23E-β	SSLQAGSWLMHYLRGGDSR	β	Estradiol

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24E- $\beta$	SRPEGSSWLLHYLSR	$\beta$	Estradiol
25E- $\beta$	SSRTLLEHYLLGGS	$\beta$	Estradiol
26E- $\beta$	SRWWLDDHELLLYSSR	$\beta$	Estradiol
27E- $\beta$	SSRTLYCHLTSSNPEWCSR	$\beta$	Estradiol
28E- $\beta$	SSRLMCWLGSADTSHCSR	$\beta$	Estradiol
29E- $\beta$	SSYDWQCPSWYCPAPPSSR	$\beta$	Estradiol
30E- $\beta$	SSTTWRCPEWYCGSR	$\beta$	Estradiol
31E- $\beta$	SSWDFRVPWWYNNR	$\beta$	Estradiol
32E- $\beta$	SSQWQAPWWYIDASR	$\beta$	Estradiol
33E- $\beta$	SSRPSFTIPWWFDDPSRSR	$\beta$	Estradiol
34E- $\beta$	SSYEIPK WALQWLSR	$\beta$	Estradiol
35E- $\beta$	SSLDLSQFPMTASFLRESR	$\beta$	Estradiol

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Table 10: Panel Peptides for Example 2

	$\alpha/\beta$ I, SSNHQSSRLIELLSR (AB1) [17 $\beta$ -estradiol]
	$\alpha/\beta$ II, SAPRATISHYLMGG (AB2) [no modulator]
	$\alpha/\beta$ III, SSWDMHQFFWEGVSR (AB3) [4-OH tamoxifen]
5	$\alpha/\beta$ IV, SRLPPSVFSMCGSEVCLSR (AB4) [same]
	$\alpha/\beta$ V, SSPGSREWFVKDMLSR (AB5) [same]
	$\alpha$ I, SSEYCFYWDSAHCSSR (A1) [17 $\beta$ -estradiol]
	$\alpha$ II, SSLTSRDFGSWYASR (A2) [17 $\beta$ -estradiol]
	$\alpha$ III, SRTWESPLGTWEWSR (A3) [no modulator]
10	$\beta$ I, SREWEDGFGRWLSR (B1) [4-OH tamoxifen]
	$\beta$ II, SSLDLSQFPMTASFLRESR (B2) [17 $\beta$ -estradiol]
	$\beta$ III, SSEACVGRWMLCEQLGVSR. (B3) [no modulator]

Alternative name parenthesized. Modulator used to isolate peptide in brackets.

**Table 11: ER $\alpha$  Binding Activity, in Presence of SERMs or Buffer, of Peptides Isolated on ER $\alpha$ -ERE**

SERM's peptides	buffer	estra-	estriol	prem-	tamox-	nafox-	clomi-	ralox-	ICI	16 $\alpha$	DES	progest
1R	7+	7+	7+	7+	7+	7+	7+	7+	7+	7+	7+	7+
2R	7+	7+	7+	7+	7+	7+	7+	7+	6+	7+	7+	5+
3B	6+	2+	3+	3+	2+	2+	2+	2+	2+	2+	2+	4+
4B	5+	3+	4+	4+	3+	3+	3+	3+	3+	3+	3+	4+
5B	7+	4+	5+	4+	2+	2+	3+	3+	3+	3+	3+	5+
6B	7+	7+	7+	7+	4+	5+	5+	4+	4+	7+	6+	7+
1B	7+	7+	6+	7+	6+	6+	6+	5+	6+	6+	6+	6+
7B	5+	3+	3+	4+	4+	3+	3+	3+	2+	3+	3+	4+
10B	7+	7+	7+	7+	7+	7+	7+	7+	7+	7+	7+	7+
9B	7+	6+	7+	7+	6+	7+	7+	5+	6+	6+	6+	7+
1E	2+	5+	4+	5+	3+	3+	4+	3+	3+	3+	3+	1+
2E	2+	4+	4+	5+	2+	2+	2+	2+	2+	3+	3+	2+
3E	3+	5+	4+	5+	3+	2+	3+	2+	3+	4+	4+	3+
9E	4+	7+	6+	7+	5+	5+	5+	5+	5+	6+	6+	5+
11E	3+	6+	6+	4+	4+	3+	4+	4+	3+	4+	4+	4+
4E	5+	7+	7+	7+	5+	4+	5+	5+	5+	6+	6+	5+
5E	3+	5+	6+	6+	3+	3+	3+	3+	3+	4+	4+	3+
6E	2+	4+	4+	4+	2+	2+	2+	2+	2+	3+	3+	2+

14E	3+	6+	6+	6+	2+	2+	2+	2+	2+	2+	4+	4+	3+
7E	2+	5+	4+	4+	2+	2+	2+	3+	2+	2+	3+	3+	2+
8E	4+	7+	6+	6+	4+	4+	4+	4+	4+	4+	5+	4+	4+
15E	2+	5+	4+	3+	2+	2+	2+	2+	2+	2+	3+	3+	3+
10E	6+	7+	7+	6+	6+	6+	6+	6+	6+	6+	7+	7+	5+
13E	3+	7+	5+	5+	3+	3+	3+	3+	3+	3+	5+	5+	4+
16E	7+	7+	7+	7+	7+	7+	7+	7+	7+	7+	7+	7+	7+
17E	2+	4+	3+	4+	2+	2+	2+	2+	2+	2+	2+	2+	2+
18E	3+	6+	5+	5+	3+	3+	3+	3+	3+	3+	3+	v	3+
E1-1	5+	7+	7+	6+	5+	6+	6+	6+	5+	5+	5+	5+	5+
E1-3	3+	7+	7+	6+	3+	3+	3+	3+	3+	2+	3+	4+	3+
E1-4	3+	6+	6+	4+	3+	3+	3+	3+	3+	2+	3+	3+	3+
E1-7	3+	6+	6+	5+	2+	2+	2+	2+	2+	2+	2+	3+	2+
I4-10	3+	6+	6+	6+	4+	5+	4+	4+	4+	5+	3+	4+	4+
T3-1	2+	2+	2+	2+	5+	4+	4+	4+	3+	2+	2+	2+	2+
T1-10	3+	3+	2+	3+	5+	4+	4+	4+	3+	2+	2+	2+	2+
T2-10	3+	3+	3+	3+	6+	4+	4+	5+	4+	3+	3+	3+	3+
T3-11	7+	5+	5+	6+	7+	6+	7+	7+	5+	5+	6+	5+	5+

**Table 12:  $Erb$  Binding Activity, in Presence of Buffer or SERMS,  
of Peptides Isolated on  $ER\alpha$ -ERE**

SERM's >	buffer	estra- diol	estriol	prem- arin	tamox- ifen	nafox- idine	clomi- phene	ralox- ifene	ICI	HPTE	$16_{-}\alpha$ OH estr- one	4-OH estr- one
peptides												
1R	0	1+	0	2+	0	1+	2+	1+	1+	1+	2+	2+
2R	0	0	0	1+	0	0	0	0	0	0	0	1+
3B	0	0	0	1+	0	0	0	0	0	0	0	0
4B	0	0	0	1+	0	0	0	0	0	0	0	0
5B	0	0	0	1+	0	0	1+	1+	0	1+	1+	1+
6B	6+	3+	5+	5+	1+	4+	1+	2+	1+	4+	4+	4+
1B	0	0	0	2+	1+	1+	0	1+	1+	1+	2+	1+
7B	0	0	0	2+	0	0	0	0	0	0	0	0
10B	0	0	1+	1+	1+	2+	2+	2+	1+	2+	2+	2+
9B	0	1+	1+	2+	2+	2+	2+	2+	2+	2+	2+	1+
1E	0	0	0	2+	0	0	1+	1+	1+	1+	1+	1+
2E	1+	7+	7+	5+	1+	2+	2+	2+	2+	2+	4+	2+
3E	1+	7+	7+	5+	1+	2+	2+	2+	1+	2+	4+	2+
9E	2+	6+	6+	5+	1+	1+	1+	1+	1+	2+	3+	2+
11E	0	7+	6+	5+	2+	3+	2+	3+	2+	3+	5+	3+

4E	0	4+	4+	5+	2+	2+	2+	2+	2+	2+	2+	3+	2+
5E	0	5+	6+	5+	0	1+	1+	1+	1+	1+	1+	3+	2+
6E	0	5+	5+	4+	0	1+	1+	1+	1+	0	1+	2+	1+
14E	4+	7+	7+	7+	1+	2+	2+	1+	1+	1+	3+	6+	4+
7E	1+	7+	7+	6+	0	1+	1+	1+	1+	1+	2+	4+	2+
8E	2+	7+	7+	7+	1+	2+	2+	1+	1+	1+	2+	4+	3+
15E	1+	7+	7+	6+	0	1+	0	0	0	0	1+	3+	1+
10E	0	7+	6+	5+	1+	1+	1+	1+	1+	1+	1+	3+	2+
13E	0	3+	3+	4+	1+	1+	0	1+	0	0	1+	2+	1+
16E	0	1+	1+	2+	1+	1+	1+	1+	1+	1+	1+	2+	1+
17E	0	4+	3+	3+	0	0	0	0	0	1+	0	1+	0
18E	1+	6+	6+	5+	0	1+	1+	1+	1+	1+	1+	3+	2+
E1-1	5+	7+	7+	7+	3+	6+	7+	4+	3+	3+	5+	5+	5+
E1-3	7+	7+	7+	7+	1+	4+	3+	2+	2+	2+	4+	3+	4+
E1-4	7+	7+	7+	7+	1+	4+	2+	2+	2+	+	4+	4+	4+
E1-7	7+	7+	7+	7+	2+	7+	4+	4+	2+	2+	6+	6+	6+
I4-10	1+	1+	2+	2+	2+	2+	1+	1+	1+	1+	1+	1+	1+
T3-1	2+	2+	2+	3+	3+	4+	4+	3+	3+	3+	3+	3+	3+
T1-10	5+	3+	3+	3+	7+	7+	7+	6+	2+	2+	3+	3+	3+
T2-10	3+	3+	3+	3+	7+	7+	7+	5+	2+	2+	3+	3+	3+
T3-11	4+	2+	3+	3+	7+	7+	7+	6+	2+	2+	4+	3+	3+



**Table 13: Binding of New Erβ-ERE Peptides, in presence of Buffer or SERMS, to Erα or Erβ Receptors**

Peptide	Receptor Form bound to and Modulator present						
	ERβ and buffer	Estradiol	ERβ and tamoxifen	buffer	ERα and estradiol	ERα and tamoxifen	
8b-β	7+	7+	1+	1+	4+	2+	
7b-β	7+	7+	2+	2+	4+	3+	
13b-β	7+	7+	2+	2+	2+	2+	
14b-β	7+	7+	7+	2+	2+	3+	
17b-β	5+	2+	4+	2+	2+	3+	
24b-β	7+	7+	7+	2+	2+	3+	
25b-β	7+	6+	7+	3+	3+	3+	
11b-β	7+	7+	7+	1+	1+	1+	
16b-β	7+	4+	5+	2+	3+	3+	
18b-β	7+	7+	7+	2+	2+	2+	
19b-β	7+	6+	7+	5+	4+	4+	
20b-β	7+	4+	5+	4+	3+	4+	
3b-β	5+	2+	++	5+	3+	3+	
4b-β	7+	3+	3+	4+	3+	3+	
5b-β	7+	6+	7+	6+	6+	7+	

6b- $\beta$	6+	2+	2+	2+	3+
1b- $\beta$	7+	7+	2+	7+	3+
21b- $\beta$	7+	7+	3+	7+	3+
23b- $\beta$	7+	7+	3+	6+	3+
1t- $\beta$	3+	2+	7+	3+	7+
9t- $\beta$	3+	2+	7+	3+	6+
16t- $\beta$	7+	5+	7+	6+	7+
7t- $\beta$	7+	7+	7+	2+	2+
8t- $\beta$	7+	5+	5+	2+	2+
12t- $\beta$	7+	7+	7+	1+	1+
13t- $\beta$	7+	7+	7+	2+	4+
2t- $\beta$	7+	6+	7+	2+	3+
17t- $\beta$	7+	7+	7+	2+	2+
20t- $\beta$	7+	6+	7+	2+	3+
6t- $\beta$	7+	4+	7+	4+	6+
22t- $\beta$	5+	4+	7+	4+	6+
23t- $\beta$	6+	6+	7+	4+	6+
5t- $\beta$	2+	2+	7+	2+	5+
15t- $\beta$	6+	2+	7+	2+	5+
14t- $\beta$	3+	2+	7+	2+	7+
10t- $\beta$	4+	2+	7+	3+	6+
21t- $\beta$	3+	3+	7+	3+	6+

18t- $\beta$	6+	3+	7+	7+	6+
4t- $\beta$	7+	7+	7+	2+	7+
1E- $\beta$	5+	7+	3+	5+	7+
2E- $\beta$	4+	7+	2+	6+	7+
3E- $\beta$	3+	6+	1+	4+	5+
4E- $\beta$	5+	7+	3+	6+	5+
5E- $\beta$	5+	7+	2+	4+	6+
6E- $\beta$	3+	7+	1+	3+	5+
7E- $\beta$	5+	6+	1+	4+	6+
8E- $\beta$	5+	7+	1+	4+	6+
9E- $\beta$	4+	7+	1+	3+	4+
10E- $\beta$	4+	7+	2+	5+	6+
11E- $\beta$	5+	6+	4+	6+	5+
12E- $\beta$	4+	7+	1+	2+	2+
13E- $\beta$	2+	6+	1+	5+	4+
14E- $\beta$	5+	7+	2+	5+	7+
15E- $\beta$	4+	7+	V	6+	6+

16E- $\beta$	6+	7+	2+	3+	6+	3+
17E- $\beta$	3+	7+	1+	5+	4+	4+
18E- $\beta$	4+	7+	1+	5+	6+	5+
19E- $\beta$	4+	7+	2+	4+	5+	3+
20E- $\beta$	3+	7+	2+	5+	5+	4+
21E- $\beta$	3+	7+	1+	4+	5+	3+
22E- $\beta$	5+	7+	2+	4+	5+	3+
23E- $\beta$	5+	7+	2+	6+	6+	5+
24E- $\beta$	5+	7+	2+	5+	6+	4+
25E- $\beta$	3+	7+	2+	5+	5+	4+
26E- $\beta$	5+	7+	2+	6+	4+	5+
27E- $\beta$	6+	7+	2+	4+	6+	3+
28E- $\beta$	3+	7+	2+	4+	3+	3+
29E- $\beta$	7+	7+	5+	3+	4+	3+
30E- $\beta$	6+	7+	3+	4+	3+	3+
31E- $\beta$	3+	7+	2+	3+	2+	3+
32E- $\beta$	5+	7+	2+	5+	4+	4+

33E- $\beta$	5+	7+	2+	3+	3+	2+
34E- $\beta$	4+	6+	2+	5+	3+	4+
35E- $\beta$	2+	6+	1+	2+	2+	2+

Table 14A: Class Specific Fingerprint on ER $\alpha$ 

Class	Peptide	buffer	Estra- diol	Es- triol	Prem- arin	Modulator (SERM) present during binding									
						Tamox- ifen	Naf oxi- dine	Clomi phene	Ral oxif- ene	ICI 182,7 80	16a- OH Estr one	DES	Pro- gest- erone		
$\alpha/\beta$ I $\alpha/\beta$ II $\alpha/\beta$ III $\alpha/\beta$ IV $\alpha/\beta$ V	#4	1+	6+	4+	2+	1+	1+	1+	1+	1+	2+	2+	1+		
	#48 ER	7+	2+	4+	2+	1+	1+	1+	1+	1+	2+	2+	6+		
	2PT	1+	1+	1+	2+	7+	4+	6+	4+	1+	2+	1+	2+		
	9T $\beta$	1+	1+	1+	1+	6+	4+	4+	2+	0	1+	1+	1+		
	14T $\beta$	1+	1+	1+	1+	1+	1+	2+	1+	1+	1+	1+	1+		
$\alpha$ I $\alpha$ II $\alpha$ III	#33	7+	7+	7+	6+	7+	7+	7+	7+	7+	6+	6+	6+		
	#5	1+	6+	5+	6+	5+	4+	5+	4+	6+	5+	4+	1+		
	#13 ER	5+	2+	2+	2+	6+	2+	5+	2+	2+	2+	3+	4+		

Table 14B: Class Specific Fingerprint on ER $\beta$ 

Class	Peptide	Modulator (SERM) present during binding										DES	Pro- gest- erone
		buffer	Estra- diol	Es- triol	Prem- arin	4-OH Tamox- ifen	Naf oxi- dine	Clomi phene	Ral- oxif- ene	ICI 182,7 80	16a- OH Estr one		
$\alpha/\beta$ I	#4	2+	7+	7+	6+	0	1+	0	0	0	5+	5+	1+
$\alpha/\beta$ II	#48 ER	7+	2+	6+	4+	1+	4+	1+	4+	2+	3+	3+	6+
$\alpha/\beta$ III	2PT	2+	1+	1+	1+	7+	3+	5+	6+	1+	1+	1+	1+
$\alpha/\beta$ IV	9T $\beta$	2+	1+	1+	1+	7+	5	5+	4+	1+	1+	1+	1+
$\alpha/\beta$ V	14T $\beta$	1+	1+	1+	1+	7+	3+	5+	2+	0	1+	1+	1+
$\beta$ I	4T $\beta$	6+	3+	2+	7+	7+	4+	3+	4+	0	2+	4+	5+
$\beta$ I	35E $\beta$	1+	5+	6+	4+	0	0	0	0	0	3+	3+	0
$\beta$ III	12B $\beta$	7+	7+	7+	7+	1+	5	3+	3+	1+	7+	7+	5+

Notes to Table 14:

Fingerprint analysis of estrogen receptor modulators on (A) ER  $\alpha$  and (B) ER  $\beta$ . Immobilized ER was incubated with estradiol (1  $\mu$ M), estriol (1  $\mu$ M), premarin (10  $\mu$ M), 4-OH  
5 tamoxifen (1  $\mu$ M), nafoxidine (10  $\mu$ M), clomiphene (10  $\mu$ M), raloxifene (1  $\mu$ M), ICI 182,780 (1  $\mu$ M), 16 $\alpha$ -OH estrone (10  $\mu$ M), DES (1  $\mu$ M) or progesterone (1  $\mu$ M). Phage ELISAs were conducted as described.

Table 15a Binding of the peptide probes to ER $\alpha$  in the presence of modulators

Peptide Probe	$\alpha/\beta$ I		$\alpha/\beta$ III		$\alpha/\beta$ IV		$\alpha/\beta$ V		$\alpha$ II	
	Equiv. <sup>a</sup>	EC50 <sup>b</sup>	Equiv.	EC50	Equiv.	EC50	Equiv.	EC50	Equiv.	EC50
Buffer	0		0		0		0		0	
17 $\beta$ -Estradiol	100	8.0	-66	18.0	-43	8.1	0		100	17.5
17 $\alpha$ -Estradiol	53	10.0	-61	88.0	-54	5.9	0		80	9.6
Estrinol	65	8.1	-59	19.2	-28	44.9	0		62	11.8
4-OH Tamoxifen	0		100	54.9	100	59.6	100	30.9	38	41.7
Nafoxidine	0		23	292.1	13	372.2	0		32	39.0
Clomiphene	0		37	143.2	19	708.5	19	282.1	56	118.9
Raloxifene	0		51	49.2	0		0		44	41.7
ICI 182,780	0		-100	25.8	-100	24.7	0		56	28.5
Diethylstilbestrol	71	13.4	-53	29.7	0		0		69	15.8
GW7604	0		0		0		0		35	8.4

<sup>a</sup>Equivalency may be positive or negative. These are both expressed in relative (percentage terms) but the positive and negative standards (100 and -100% marks) are set differently. Thus, the positive and negative values are scaled differently. Positive equivalency is defined as the maximum stimulation achieved with a given compound as a percentage of the maximum stimulation achieved with the positive modulator used for isolation of a given peptide probe (see Table 10). Negative values indicates that an increase in the concentration of a compound results in a reduction of the binding of the peptide probe as compared to the binding of the probe in buffer. These are expressed as a percentage of the reduction by ICI 182,780. For  $\alpha$ II, ICI 182,780 acts as an agonist, and its equivalency is therefore stated as a percentage of the reference modulator  $\beta$  estradiol. Results for  $\alpha$ III were zero in all cases. <sup>b</sup>EC50 is defined as the concentration in nanomolar of a given compound required to achieve fifty percent of the maximal signal for that compound.



Table 15b Binding of the peptide probes to ER $\beta$  in the presence of modulators

Peptide Probe	$\alpha/\beta$ I		$\alpha/\beta$ III		$\alpha/\beta$ IV		$\alpha/\beta$ V		$\beta$ I		$\beta$ III	
	Equiv. <sup>a</sup>	EC50 <sup>b</sup>	Equiv.	EC50	Equiv.	EC50	Equiv.	EC50	Equiv.	EC50	Equiv.	EC50
Buffer	0		0		0		0		0		0	
17 $\beta$ -Estradiol	100	21.8	-71 <sup>c</sup>	5.7	-84	26.7	0		-69	12.8	100	17.0
17 $\alpha$ -Estradiol	44	8.8	-78	7.1	-82	12.9	0		-74	10.1	42	6.7
Estrinol	81	19.5	-57	15.8	-75	12.4	0		-96	20.7	77	11.7
4-OH Tamoxifen			100	37.3	100	179.8	100	50.0	100	20.6	-100	34.4
Nafoxidine			27	231.7	0		0		-44	320.5	0	
Clomiphene			34	82.2	0		13	149.8	-62	135.1	-61	122.5
Raloxifene			77	90.1	0		0		-53	89.9	-71	156.2
ICI 182,780			-100	18.1	-100	35.3	0		-100	28.9	-100	48.4
Diethylstilbesterol	68	33.9	-78	14.5	-96	17.8	0		-59	11.1	86	25.4
GW 7604	0		-86	4.2	74	3050.1	0		159	3.3	-106	7.7

<sup>a</sup>Positive equivalency is defined as the maximum stimulation achieved with a given compound as a percentage of the maximum stimulation achieved with the modulator used for isolation of a given peptide probe. The equivalency numbers for these reference modulators are bolded. See also Table 10. Negative values indicate that an increase in the concentration of a compound results in a reduction of the binding of the peptide probe as compared to the binding of the probe in buffer. These negative values are expressed as a percentage of the reduction by ICI 182,780, so ICI 182,780 was scored -100 by definition, and is also bolded. Results for  $\alpha/\beta$ II were zero in all cases. <sup>b</sup>EC50 is defined as the concentration in nanomolar of a given compound required to achieve fifty percent of the maximal signal for that compound.

**Table 99**  
**Peptide Interactions with ER Alpha**

Peptide	No Addition		Estradiol		4-hydroxy Tamoxifen		Tamoxifen Citrate	
	value	standard deviation	value	standard deviation	value	standard deviation	value	standard deviation
F6	30983	7961	38883	2899	20267	3313	11947	460
A2	1224	8	30983	2993	20100	2146	8933	1299
AB3	381	16	605	64	3194	86	600	38
AB5	548	16	648	44	4128	25	1005	36

Values are in beta-galactosidase units (MOD/min/mg)

Table 100

<b>A</b>		S R A G L L S D L L E G K S R
		S S R S L L R D L L M V D S R
		S S N K L L Y N L L K M E S R
5		S S K S L L L N L L S T P S R
	H S F P R E	S L L V R L L Q G G
		S R L E M L L R S E T D F S R
		S R L E E L L K W G S V T S R
10		S R L E Q L L K E E F S Y S R
		S R L E Q L L R S E P D F S R
		S R L E D L L R A P F T T S R
		S R L E S L L R F G Q L D S R
		S S R L L S L L V G D F N S R
15		S R L E E L L L G T N R D S R
		S R L E E L L L M D F W R S R
		S R L K E L L L L P T D L S R
		S R L E C L L E G R L N C S R
		S S K L Y C L L D E S Y C S R
		S R L S C L L M G F E D C S R
20		S S K L I R L L T S D E E L S R
		S S R L M E L L Q E G Q G W S R
		S S N H Q S S R L I E L L S R
		S S R L W Q L L A S T D T S R
		S S K L W Q L L S S P I D S R
25		S R L V A L L K S P W S V S R
		S S N S M L W K L L A A P S R
		S S K T L W R L L E G E R S R
		S R A G P V L W G L L S E S R
30		S R S P I L T H L L S L G S R
		S S T G I L W K L L T A E S R
		S S H G I L W R L L S E G S R
<b>B</b>		
		K L V Q L L T T T A E
35	SRC1a	I L H R L L Q E G S P
		L L R Y L L D K D E K
		L L Q Q L L T E
		Q L S E L L R G G S G
		Q L V L L L H A H K C
40		Y L E G L L M H Q A A
		L L A S L L Q S E S S
		H L K T L L K K S K V
		Q L A L L L S S E A H
		L L L H L L K S Q T I
		L L Q L L L G H K N E
45	RIP140	V L Q L L L G N P K G
		L L S R L L R Q N Q D
		V L K Q L L L S E N C

Table 101

	ER4	SSNHQSRLELLSR
	D47	HVYQHPLLLSLLSSEHESG
	C33	HVEMHPLLMLLMESQWGA
5	D14	QEAHGPLLWNLLSRSDTDW
	F6	GHEPLTLLERLLMDDKQAV
	D22	LPYEGSLLLKLLRAPVEEV
	D48	SGWENSILYSLLSDRVSLD
	D43	AHGESSLLAWLLSGEYSSA
10	D17	GVFCDSILCQLLAHDNARL
	D41	HHNGHSILYGLLAGSDAPS
	D26	LGERASLLDMLLRQENPAW
	D40	SGWNESTLYRLLQADAFDV
	D15	PSGGSSVLEYLLTHDTSIL
15	D2	GSEPKSRLLLELLSAPVTDV
	D30	HPTHSSRLWELLMEATPTM
	D11	VESGSSRLMQLLMANDLLT
	D10	WEEHSQMLLHLLDTGEAVW
	F4	PVGEPGLLWRLLSAPVERE

References

- Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X.-Y., Sauter, G., Kallioniemi, O.-P., Trent, J. M., and Meltzer, P. S. (1997) AIB1 a steroid receptor  
5 coactivator amplified in breast and ovarian cancer. *Science* 277, 965-968.
- Chambraud, B., Berry, M., Redeuilh, G., Chambon, P., and Baulieu, E., (1990) Several regions of the human estrogen  
10 receptor are involved in the formation of receptor-heatshock protein 90 complexes. *J. Biol. Chem.* 265, 20686-20691.
- Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G., (1997) A signature motif in transcriptional co-activators  
mediates binding to nuclear receptors. *Nature*, 387 733-736.
- Kraus, W. L., McInerney, E. M., and Katzenellenbogen, B.  
15 S., (1995) Ligand-dependent, transcriptionally productive association of the amino-and carboxyl-terminal regions of a steroid hormone nuclear receptor. *Proc. Natl. Acad. Sci., USA* 92 12314-12318.
- Montano, M. M., Muller, V., Trobaugh, A., and  
20 Katzenellenbogen, B. S., (1995) The carboxy-terminal F domain of the human estrogen receptor: role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. *Mol Endocrinol.*, 9 814-825.
- Paech, K., Webb, P., Kuiper, G. G .J. M., Nilsson, S.,  
25 Gustafsson, J.-A., Kushner, P. J., and Scanlan, T. S. (1997) Differential ligand activation of estrogen receptors ER  $\alpha$  and ER  $\beta$  at AP1 sites. *Science* 277, 1508-1510.

Claims

1. A method of predicting the receptor-modulating activity of a compound which modulates the biological activity of a receptor which comprises:

- 5 (a) providing a ligand for the receptor;
- (b) screening a first combinatorial library comprising a plurality of members for the ability to bind to a receptor in at least two different reference conformations,
- 10 (c) based on said screening, providing a panel of first library members, said panel comprising members which differ with respect to their ability to binding to the receptor, depending on its conformation,
- 15 (d) screening a plurality of reference substances known to modulate the biological activity of said receptor to determine their effect on the binding of each member of said panel to said receptor, thereby obtaining a reference fingerprint for each reference substance, said fingerprint comprising a plurality of panel-based descriptors, each panel-based descriptor characterizing the effect of the reference substance on the binding of a particular panel member to said receptor, said reference fingerprint's panel based descriptors collectively characterizing the effect of the reference substance on the binding of all of the panel members, individually, to said receptor,
- 20 (e) screening a test substance of unknown activity relative to said receptor to determine its effect on the binding of each member of said panel to said receptor, thereby obtaining a test fingerprint for said test substance,
- 25 (f) comparing the test fingerprint to the reference fingerprints, and
- 30 (g) predicting the biological activity of the test substance, based on the assumption that its biological activity will be similar to that of reference substances with similar fingerprints.
- 35

2. The method of claim 1 in which at least one reference conformation is an unliganded conformation of the receptor.

3. The method of claim 2 in which at least one reference conformation is a liganded conformation of the receptor.

5 4. The method of claim 1 in which the conformations comprise a first liganded conformation induced by a first ligand and a second liganded conformation induced by a second and different ligand.

10 5. The method of claim 3 in which said panel comprises at least two of the following:

(i) a member which binds the ligand-bound receptor more strongly than it binds the unliganded receptor, and which detectably binds the unliganded receptor,

15 (ii) a member which binds the ligand-bound receptor less strongly than it binds the unliganded receptor, and

(iii) a member which binds the ligand-bound receptor about as strongly as it binds the unliganded receptor, and detectably binds both.

20 6. The method of claim 1 wherein a plurality of different ligands are used in characterizing the panel.

7. The method of claim 1 in which the biological activity of the reference substances at said receptor is known for a plurality of different tissues, so that the biological activity  
25 of the test substance in said tissues is predicted.

8. The method of claim 1 in which the receptor is a nuclear receptor.

9. The method of claim 1 in which the receptor is an estrogen receptor.

30 10. The method of claim 1 in which the receptor is a G-protein coupled receptor, a G protein, or a G protein subunit.

11. The method of claim 1 in which at least one ligand is a pharmacological agonist or antagonist of the receptor.

35 12. The method of claim 1 in which at least one conformation is induced by a natural ligand of the receptor.

13. The method of claim 1 in which at least one conformation is induced by a ligand which is not a natural ligand of the receptor.

14. The method of claim 1 in which the first combinatorial library is an oligopeptide library.

15. The method of claim 1 in which the first combinatorial library is a nucleic acid library.

5 16. The method of claim 1 in which the test substances are provided and screened in the form of a combinatorial library.

17. The method of claim 1 in which the biologically active component of said test substance is an organic compound  
10 with a molecular weight of less than 500 daltons.

18. The method of claim 1 in which screening steps (a), (d) and (e) are performed in vitro.

19. The method of claim 1 in which screening steps (a), (d) and (e) are performed in a cell-based assay which is not  
15 an assay of a whole multicellular animal or tissues and organs isolated from such an animal.

20. The method of claim 19 in which screening steps (d) and (e) are performed in a two-hybrid assay system, and the members of the panel are peptides.

20 21. The method of claim 1 in which the receptor is a glucocorticoid receptor.

22. A peptide comprising an LXXLL motif, said peptide inhibiting tamoxifen partial agonist activity.



Figure 1






























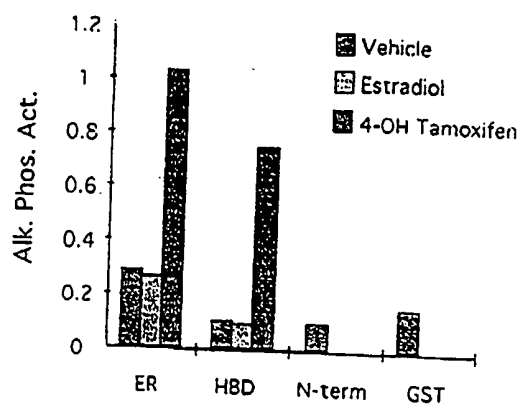
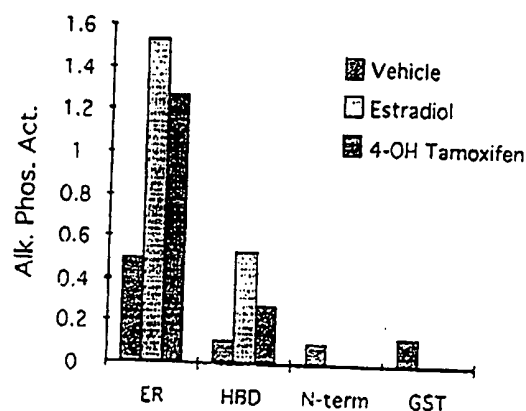
Effect 	Compound Name	Breast	Bone	Uterus	Cardiovascular	Cholesterol
	Estradiol					
	Tamoxifen					
	Raloxifene					
	ICI 182,870					
	Nafoxidine					
	Clomiphene					
	Premarin					

Figure 2  
Mapping the Binding Sites for  
ER-Interacting Peptides

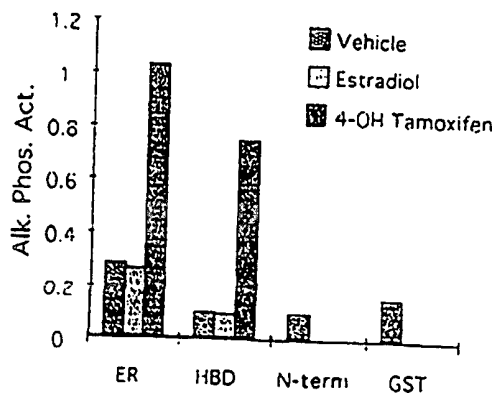
$\alpha\beta I$



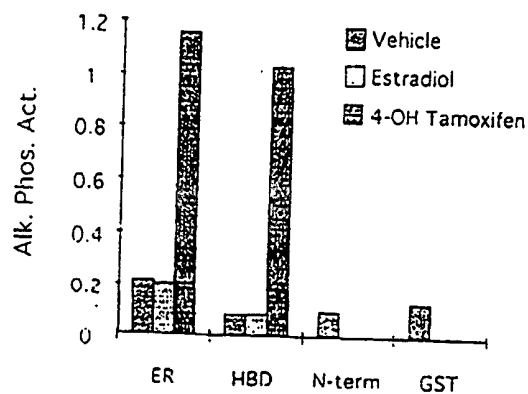
$\alpha II$



$\alpha\beta III$



$\alpha\beta V$



*Figure 3*

## Different Ligands Induce Different Structural Alterations in ER $\alpha$ and ER $\beta$

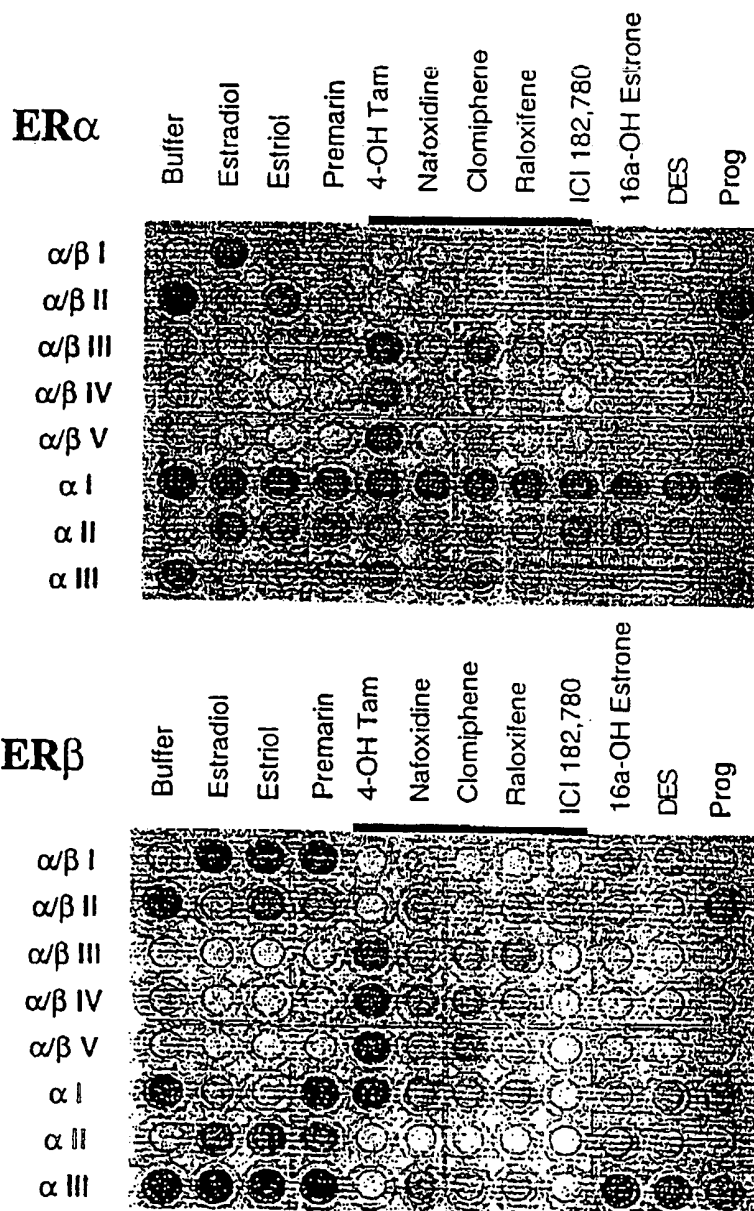
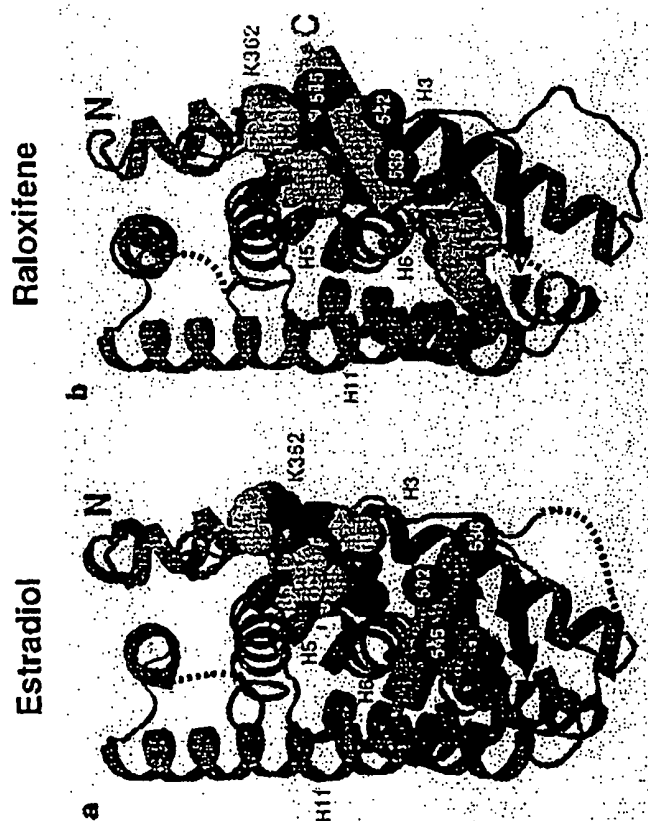


Fig. 4  
Agonists and Antagonists Induce Distinct  
Alterations in ER $\alpha$  Structure



Brzozowski et al. Nature 1997, 389:753-758

Figure 4

Fig. 5A

## ER Alpha

	buffer	estradiol	estriol	premarin	4-OH Tamoxifen	nafoxidene	clomiphene	raloxifene
$\alpha\beta$ I	1	6	4	2	1	1	1	1
$\alpha\beta$ II	7	2	4	2	1	1	1	1
$\alpha\beta$ III	1	1	1	2	7	4	6	4
$\alpha$ I	1	1	1	1	6	4	4	2
$\alpha$ II	1	1	1	1	1	1	2	1
$\alpha$ III	7	7	7	6	7	7	7	7
$\alpha$ VI	1	6	5	6	5	4	5	4
$\alpha$ V	5	2	2	2	6	2	5	2

Fig. 5B

Universal Max	19.799	19.799	19.8	19.799	19.79898987	19.79899	19.79899	19.79899
Max	17.407	16.7033	15.46	15.6205	17.40689519	15.165751	15.748016	15.45962
buffer	0	9.16515	6.557	7.87401	10.67707825	8.4852814	9.3273791	8
estradiol	9.1652	0	3	4.24264	10.19803903	6.9282032	8.4261498	6.324555
estriol	6.5574	3	0	3.31862	9.746794345	6.0827625	7.8740079	5.385165
premarin	7.874	4.24264	3.317	0	8.366600265	4.472136	6.244998	3.464102
4-OH Tamoxifen	10.677	10.198	9.747	8.3666	0	5.4772256	2.6457513	6.480741
nafoxidene	8.4853	6.9282	6.083	4.47214	5.477225575	0	3.8729833	2
clomiphene	9.3274	8.42615	7.874	6.245	2.645751311	3.8729833	0	4.358899
raloxifene	8	6.32456	4.899	3.31862	6.480740698	2.236068	4.3588989	0
ICI 182,780	8.4261	5.19615	4.472	2.23607	9.433981132	5.3851648	7.2111026	4.123106
16 $\alpha$ -OH estrone	7.2801	4.3589	3.162	1	8.306623863	4.1231056	6.164414	3
DES	6.3246	4.69042	3.317	2.44949	8.602325267	4.6904158	6.5574385	3.741657
progesterone	2	8.48528	5.916	6.78233	9.797958971	7.2111026	8.3066239	6.63325

ER Alpha

5B carb

ICI 182,780	16 $\alpha$ -OH estrone	DES	progesterone
1	2	2	1
1	2	2	6
1	2	1	2
0	1	1	1
1	1	1	1
7	6	6	6
6	5	4	1
2	2	3	4

19.79899	19.79898987	19.8	19.79898987
17.406895	15.26433752	15	16.03121954
8.4261498	7.280109889	6.32	2
5.1961524	4.358898944	4.69	8.485281374
4.472136	3.16227766	3.32	5.916079783
2.236068	1	2.45	6.782329983
9.4339811	8.306623863	8.6	9.797958971
5.3851648	4.123105626	4.69	7.211102551
7.2111026	6.164414003	6.56	8.306623863
4.2426407	3.484101615	4.12	6.708203932
0	2.449489743	3	7.549834435
2.4494897	0	1.73	6.08276253
3	1.732050808	0	5.291502622
7.5498344	6.08276253	5.29	0

Fig. 5C

Scaled alpha

	buffer	estradiol	estriol	premarin	4-OH Tam	nafoxidene	clomiphene	raloxifene
buffer	1	0.53709	0.668799	0.602303	0.460726	0.571429	0.528896	0.595939
estradiol	0.53709	1	0.848477	0.785714	0.484921	0.650073	0.574415	0.680562
estriol	0.668799	0.848477	1	0.832485	0.507713	0.692774	0.602303	0.728008
premarin	0.602303	0.785714	0.832485	1	0.577423	0.774123	0.68458	0.825036
4-OH Tamoxifen	0.460726	0.484921	0.507713	0.577423	1	0.723358	0.866369	0.672673
nafoxidene	0.571429	0.650073	0.692774	0.774123	0.723358	1	0.804385	0.898985
clomiphene	0.528896	0.574415	0.602303	0.68458	0.866369	0.804385	1	0.779842
raloxifene	0.595939	0.680562	0.752564	0.832485	0.672673	0.887062	0.779842	1
ICI 182,780	0.574415	0.737555	0.774123	0.887062	0.523512	0.728008	0.635784	0.791752
16 $\alpha$ -OH estrone	0.632299	0.779842	0.840281	0.949492	0.580452	0.791752	0.68865	0.848477
DES	0.680562	0.763098	0.832485	0.876282	0.565517	0.763098	0.668799	0.811018
progesterone	0.898985	0.571429	0.701193	0.657441	0.505128	0.635784	0.580452	0.66497

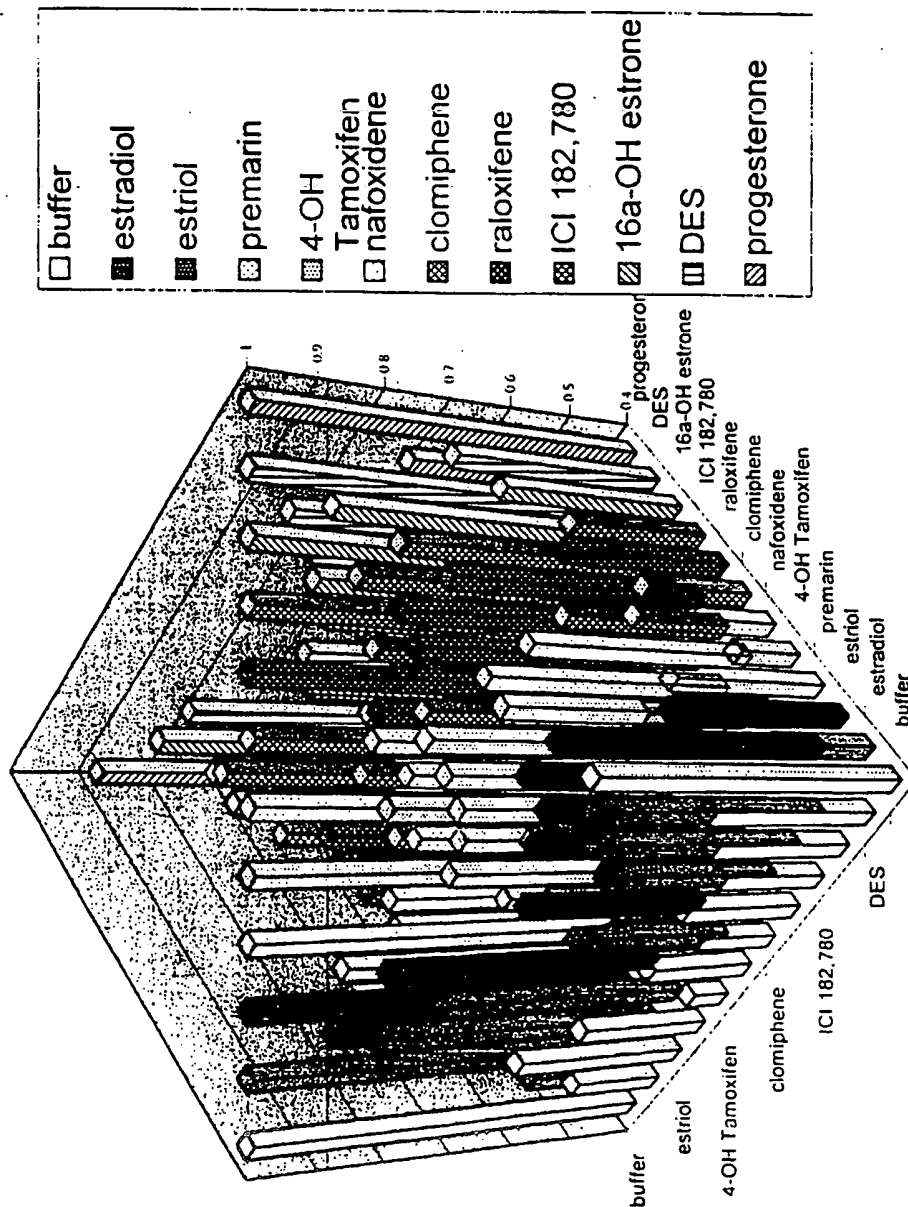
5C contd

## Scaled alpha

ICI 182,781 16 $\alpha$ -OH es DES			progesterone
0.574415	0.632299	0.680562	0.898985
0.737555	0.779842	0.763098	0.571429
0.774123	0.840281	0.832485	0.701193
0.887062	0.949492	0.876282	0.657441
0.523512	0.580452	0.565517	0.505128
0.728008	0.791752	0.763098	0.635784
0.635784	0.68865	0.668799	0.580452
0.785714	0.825036	0.791752	0.661185
1	0.876282	0.848477	0.618676
0.876282	1	0.912518	0.692774
0.848477	0.912518	1	0.732739
0.618676	0.692774	0.732739	1

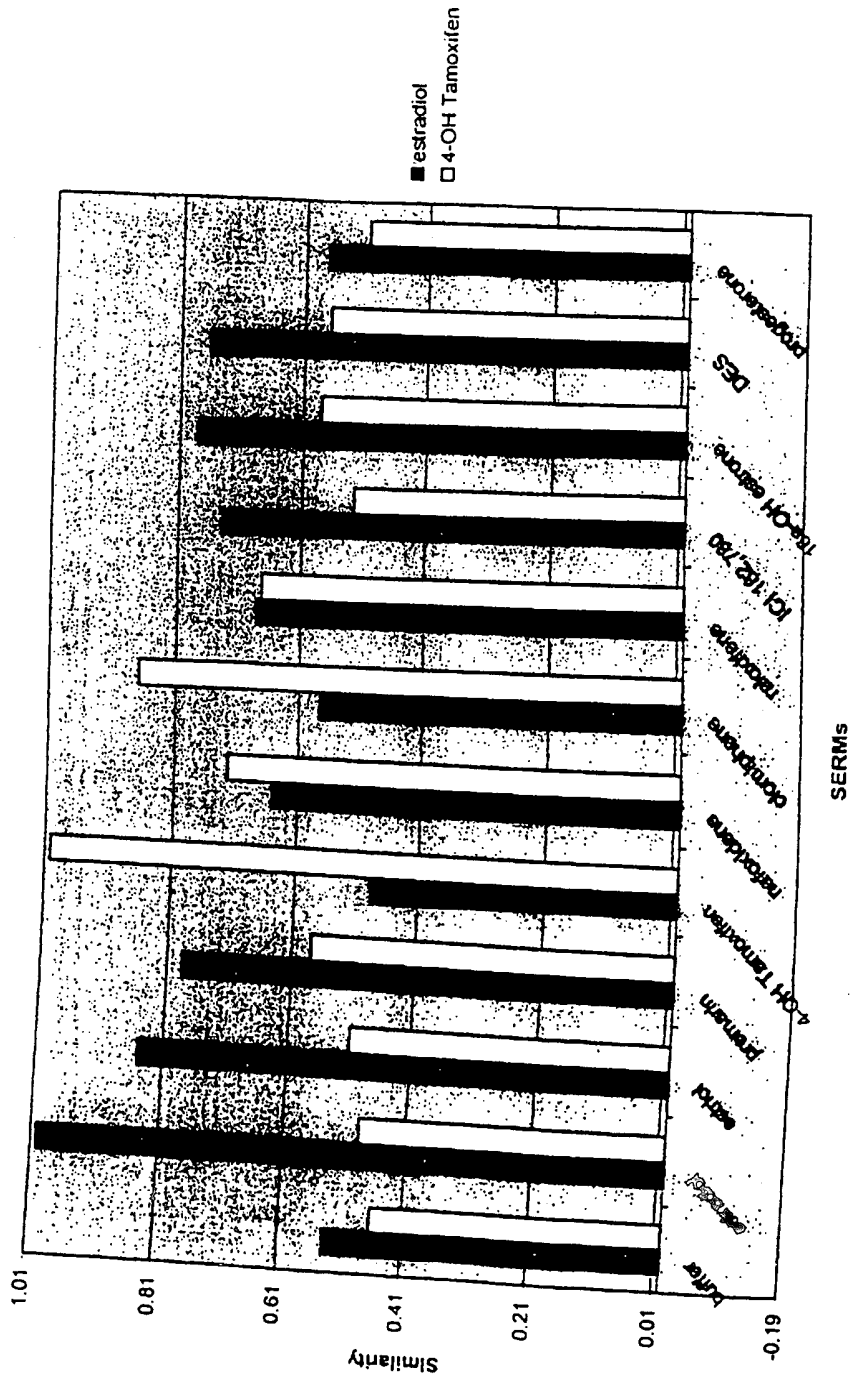


5D



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Similarities of Estradiol and 4-OH Tamoxifen to Known SERMs for ER-alpha



56

Fig. 6A

ER Beta

	buffer	estradiol	estriol	premarin	4-OH Tamoxifen	nafoxidene	clomiphene
$\alpha\beta$ I	2	7	7	6	0	1	0
$\alpha\beta$ II	7	2	6	4	1	4	1
$\alpha\beta$ III	2	1	1	1	7	3	5
$\beta$ I	2	1	1	1	7	5	5
$\beta$ II	1	1	1	1	7	3	5
$\beta$ III	6	3	2	7	7	4	3
$\beta$ VI	1	5	6	4	0	0	0
$\beta$ V	7	7	7	7	1	5	3

Universal Max			19.79899				
Max	16.76305	16.49242	17.4069	16.55295	19.13112647	14.106736	15.524175
buffer	0	8.774964	8.306624	6.082763	12.80624847	5.7445626	10
estradiol	8.774964	0	4.242641	4.690416	15.32970972	9.6953597	11.789826
estriol	8.306624	4.242641	0	5.830952	16.70329309	10.392305	13.228757
premarin	6.082763	4.690416	5.830952	0	14.31782106	8.8317609	11.874342
4-OH Tamoxifen	12.80625	15.32971	16.70329	14.31782	0	8.4261498	5.6568542
nafoxidene	5.744563	9.69536	10.3923	8.831761	8.426149773	0	4.7958315
clomiphene	10	11.78983	13.22876	11.87434	5.656854249	4.7958315	0
raloxifene	7.416198	11.40175	9.949874	8.774964	7.615773106	4	4.5825757
ICI 182,780	10.24695	10.95445	11.91638	11.91638	13.07669683	8.1240384	8.4261498
16 $\alpha$ -OH estrone	6.855655	3.162278	4.690416	5.291503	14.38749457	7.6157731	10.148892
DES	5.91808	3.162278	5.09902	3.464102	13.82027496	7.3484692	10.148892
progesterone	3.162278	9.219544	9.219544	7.28011	12.40967365	5.3851648	9.0553851

5A cont'd

ER Beta

raloxifene	ICI 182,780	16 $\alpha$ -OH estrone	DES	progesterone
0	0	5	5	1
4	2	3	3	6
6	1	1	1	1
4	1	1	1	1
2	0	1	1	1
4	0	2	4	5
0	0	3	3	0
3	1	7	7	5

14.93318	18.1383571	15.45962483	15.16575	16.70329309
7.416198	10.2469508	6.8556546	5.91608	3.16227766
11.40175	10.9544512	3.16227766	3.162278	9.219544457
12	11.9163753	4.69041576	5.09902	9.219544457
10.58301	11.9163753	5.291502622	3.464102	7.280109889
7.549834	13.0766968	14.38749457	13.82027	12.40967365
4	8.1240384	7.615773106	7.348469	5.385164807
4.582576	8.42614977	10.14889157	10.14889	9.055385138
0	7.87400787	9.486832981	9.273618	6.708203932
7.874008	0	8.717797887	9.380832	7.681145748
9.486833	8.71779789	0	2	6.8556546
9.273618	9.38083152	2	0	6.244997998
6.708204	7.68114575	6.8556546	6.244998	0

5A cont'd

ER Beta

raloxifene	ICI 182,780	16 $\alpha$ -OH estrone	DES	progesterone
0	0	5	5	1
4	2	3	3	6
6	1	1	1	1
4	1	1	1	1
2	0	1	1	1
4	0	2	4	5
0	0	3	3	0
3	1	7	7	5

14.93318	18.1383571	15.45962483	15.16575	16.70329309
7.416198	10.2469508	6.8556546	5.91608	3.16227766
11.40175	10.9544512	3.16227766	3.162278	9.219544457
12	11.9163753	4.69041576	5.09902	9.219544457
10.58301	11.9163753	5.291502622	3.464102	7.280109889
7.549834	13.0766968	14.38749457	13.82027	12.40967365
4	8.1240384	7.615773106	7.348469	5.385164807
4.582576	8.42614977	10.14889157	10.14889	9.055385138
0	7.87400787	9.486832981	9.273618	6.708203932
7.874008	0	8.717797887	9.380832	7.681145748
9.486833	8.71779789	0	2	6.8556546
9.273618	9.38083152	2	0	6.244997998
6.708204	7.68114575	6.8556546	6.244998	0

Fig. 6B

scaled beta

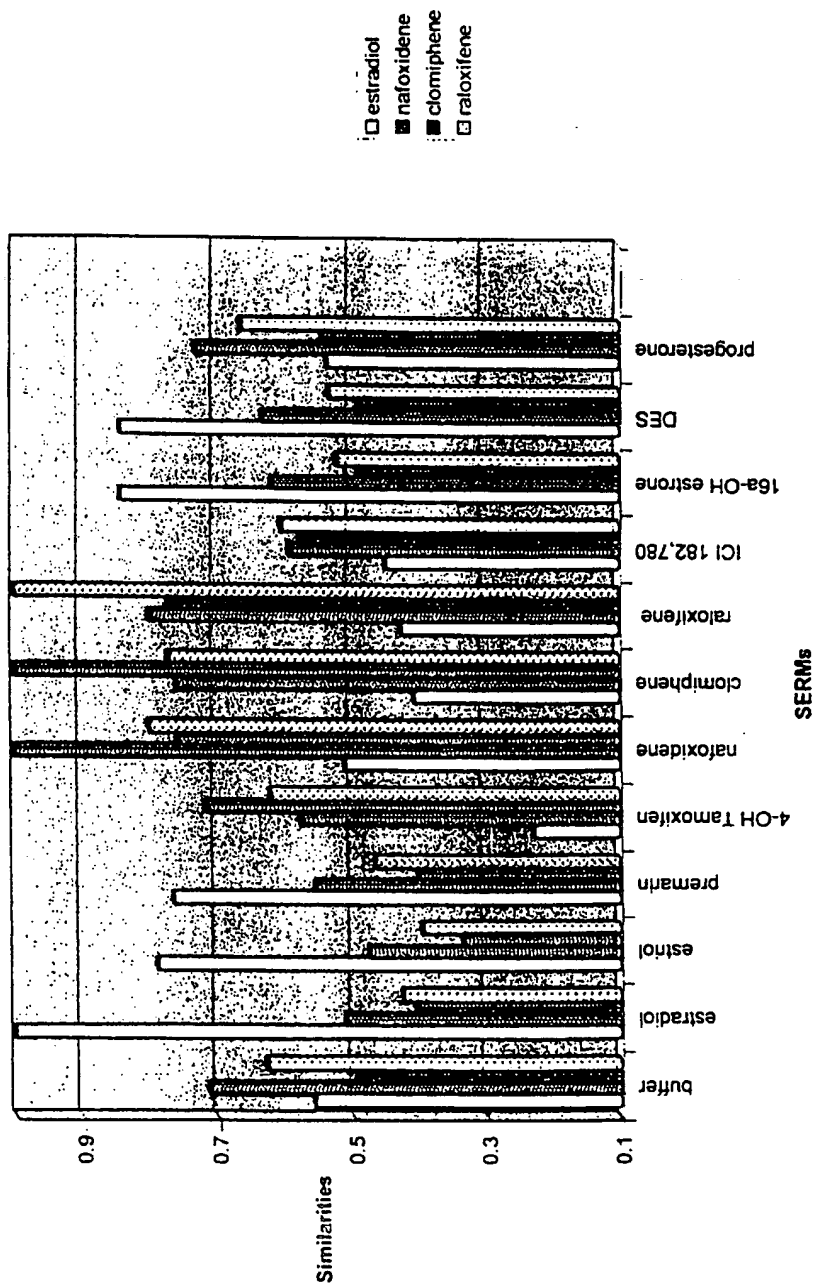
	buffer	estradiol	estriol	premarin	4-OH Tam	nafoxidene	clomiphene
buffer	1	0.556797	0.580452	0.692774098	0.353187	0.709856	0.494924
estradiol	0.556797	1	0.785714	0.763098229	0.225733	0.51031	0.404524
estriol	0.580452	0.785714	1	0.705492455	0.156356	0.475109	0.331847
premarin	0.692774	0.763098	0.705492	1	0.276841	0.553929	0.400255
4-OH Tamoxifen	0.353187	0.225733	0.156356	0.276840831	1	0.574415	0.714286
nafoxidene	0.709856	0.51031	0.475109	0.553928714	0.574415	1	0.757774
clomiphene	0.494924	0.404524	0.331847	0.400255156	0.714286	0.757774	1
raloxifene	0.625425	0.424124	0.497455	0.55679737	0.615345	0.797969	0.768545
ICI 182,780	0.482451	0.446717	0.398132	0.398132159	0.339527	0.589674	0.574415
16 $\alpha$ -OH estrone	0.653737	0.840281	0.763098	0.732738758	0.273322	0.615345	0.487404
DES	0.701193	0.840281	0.742461	0.825036447	0.301971	0.628846	0.487404
progesterone	0.840281	0.534343	0.534343	0.632298924	0.373217	0.728008	0.542634

6B contd  
scaled beta

raloxifene	ICI 182,781	16 $\alpha$ -OH es DES	progesterone
0.625425	0.482451	0.653737	0.701193
0.424124	0.446717	0.840281	0.840281
0.393908	0.398132	0.763098	0.742461
0.465478	0.398132	0.732739	0.825038
0.618676	0.339527	0.273322	0.301971
0.797969	0.589674	0.615345	0.628846
0.768545	0.574415	0.487404	0.487404
1	0.602303	0.520843	0.531612
0.602303	1	0.559685	0.526196
0.520843	0.559685	1	0.898985
0.531612	0.526196	0.898985	1
0.661185	0.612044	0.653737	0.68458

fig. 6C

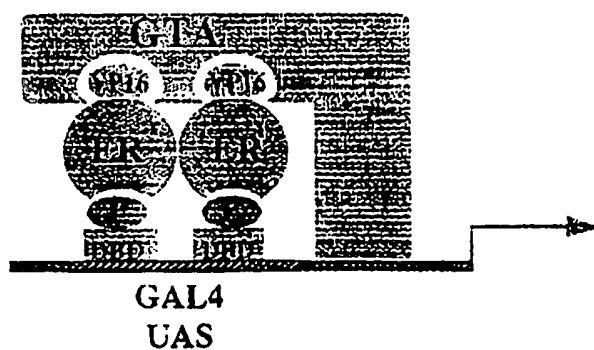
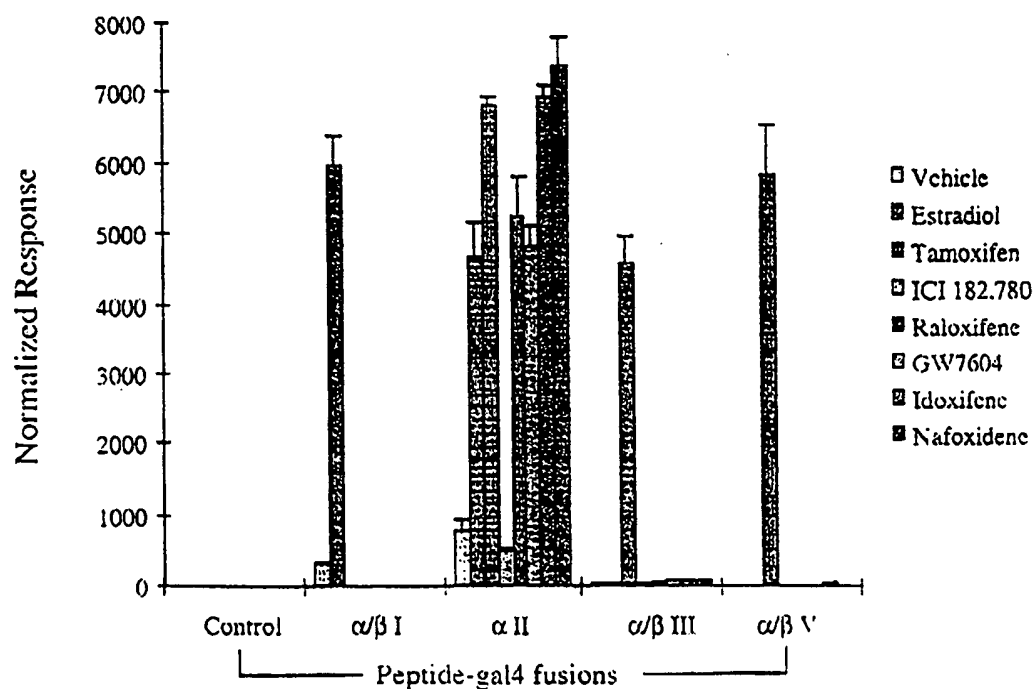
Similarities for Selected Serms for ER-beta





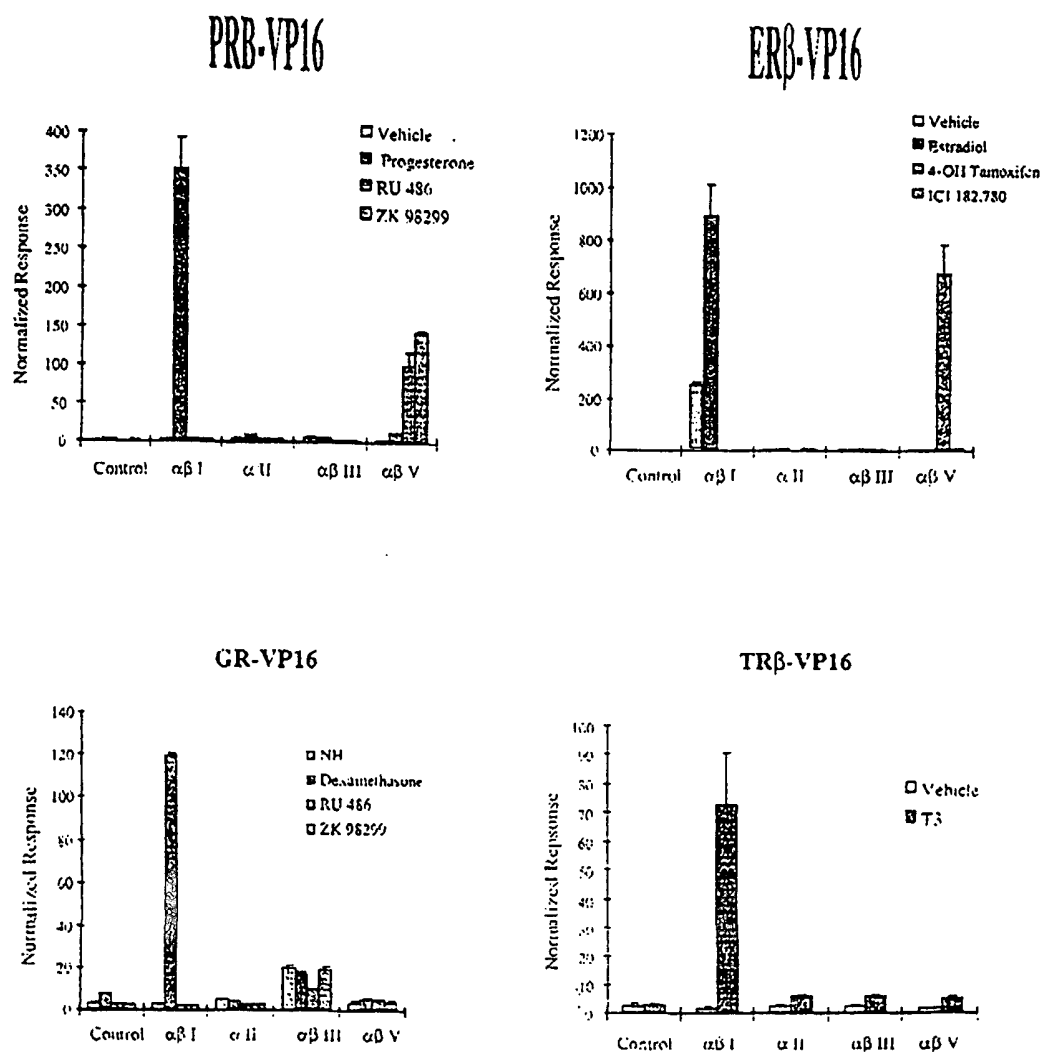
16/31

Fig. 7  
Analysis of ER-peptide interactions  
Using the mammalian two-hybrid  
System

ER $\alpha$ -VP16

17/31

Figure 8  
Analysis of the specificity of  
interaction between peptides  
and nuclear receptor family members



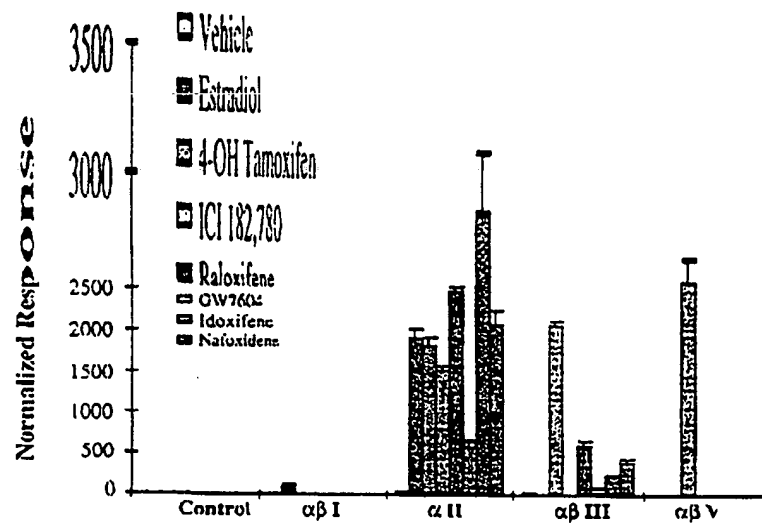
18/31

Figure 9

P<sub>1</sub> titles which interact with the Tamoxifen  
Activated ER do not require AF-2 (helix 12)

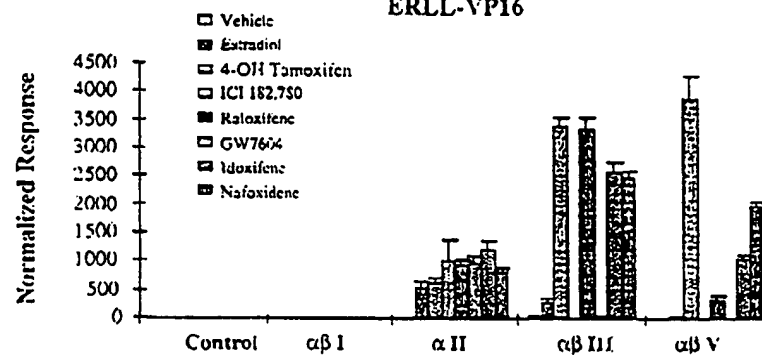
ER3X-VP16

A



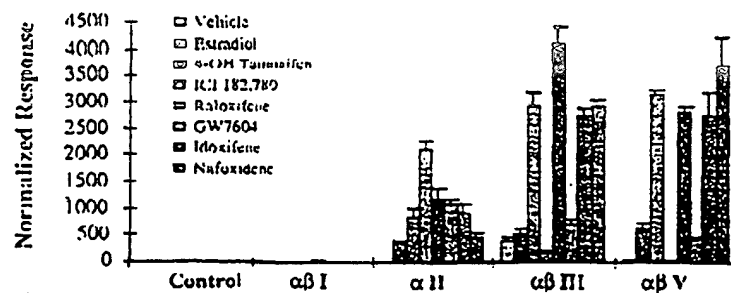
B

ERLL-VP16



C

ER535-stop

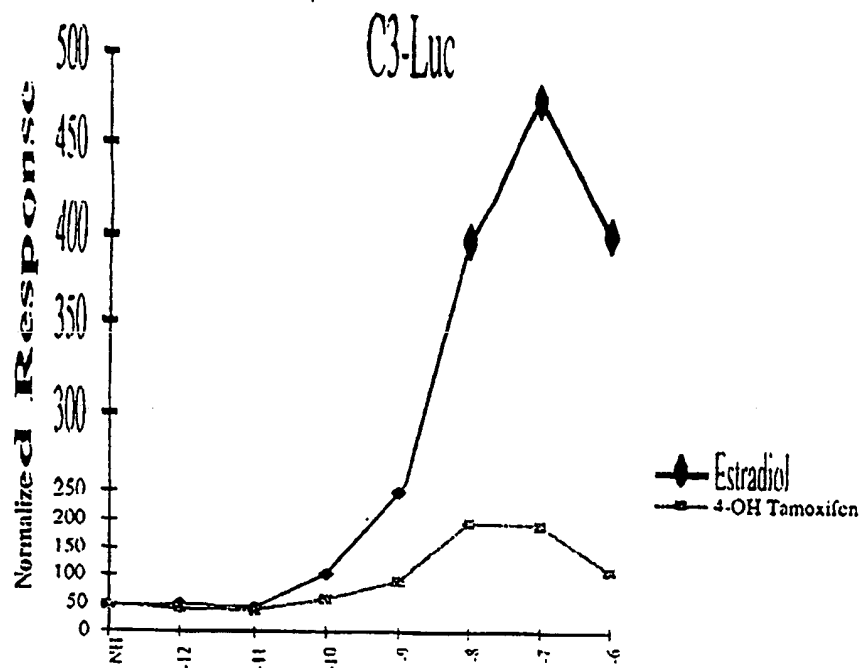


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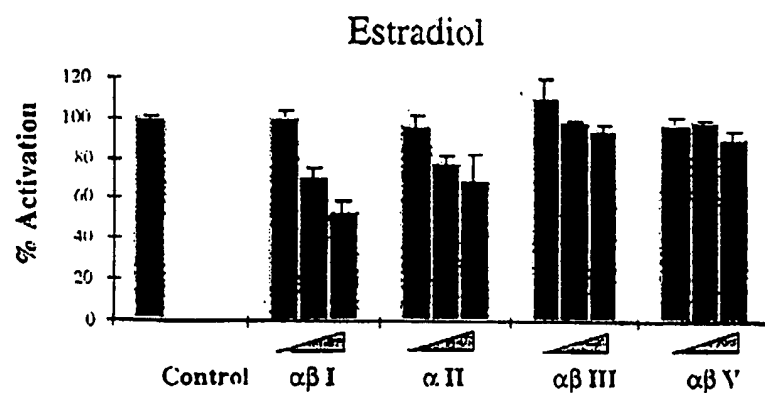
Figure 10

Disruption of ER mediated transcriptional activity by Gal4-peptide fusion proteins

A



B



C

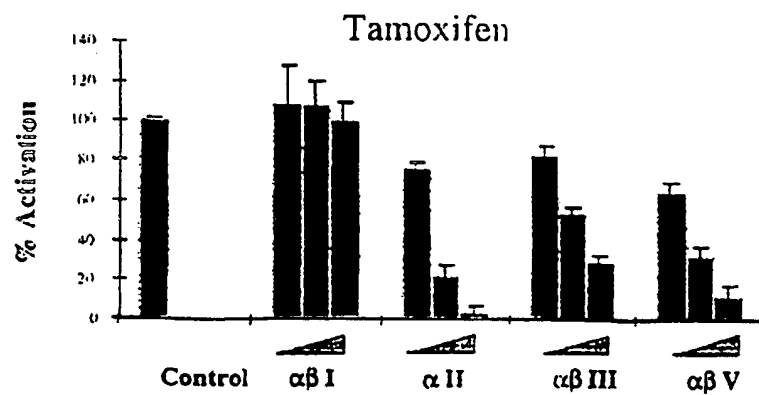


Fig. VI  
Disruption of Tamoxifen Activated  
ER Transcriptional Activity by  
 $\alpha$  II peptide is not Promoter  
Dependent

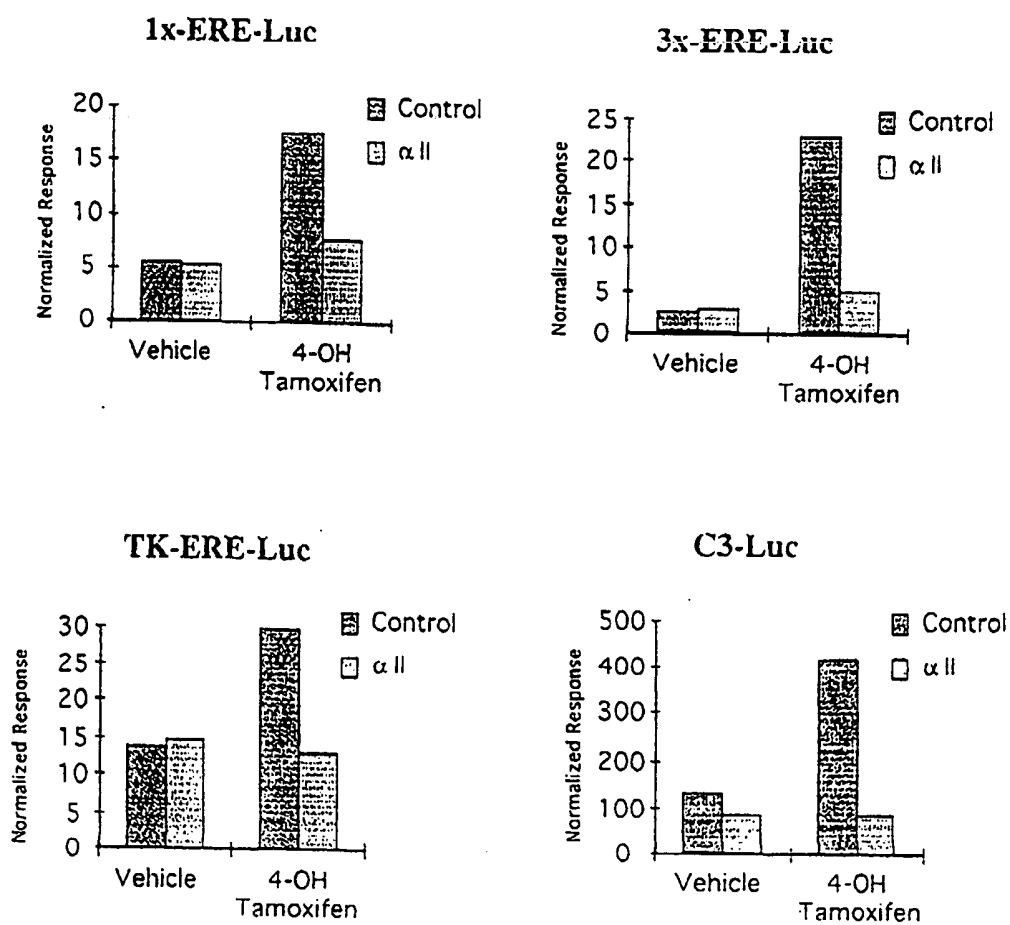
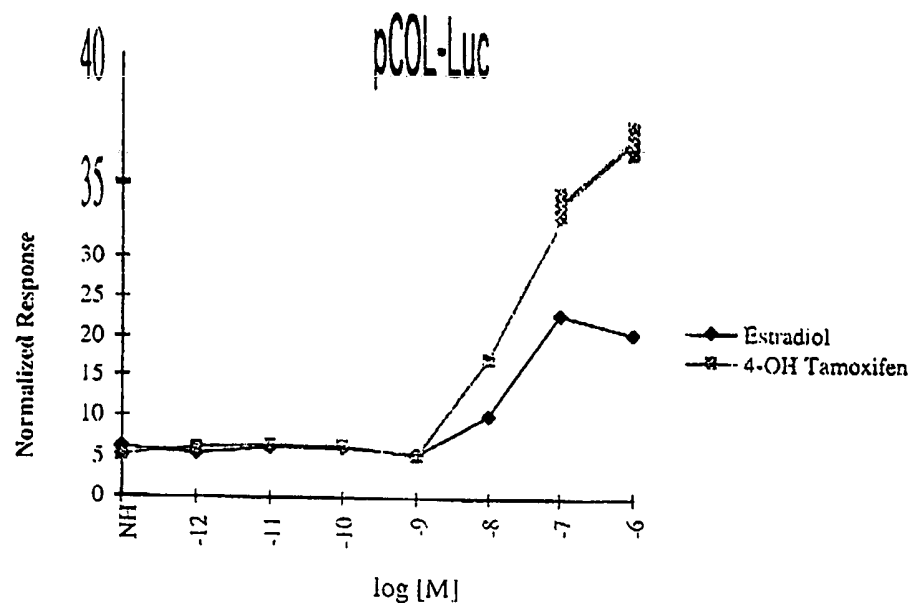


Fig. 12  
Disruption of ER mediated  
transcriptional activity through  
the AP-1 pathway by gal4  
peptide fusion proteins

A



B

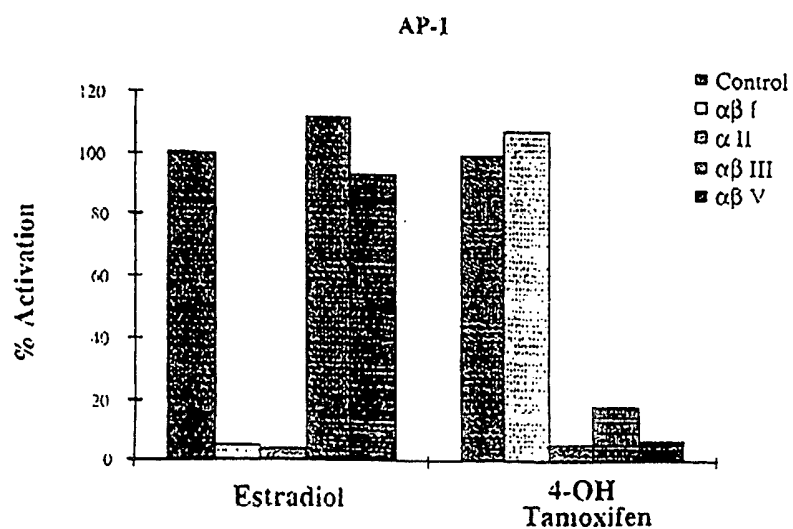


Fig. 13 Potential mechanisms to explain the activity of peptides which block tamoxifen partial agonist activity

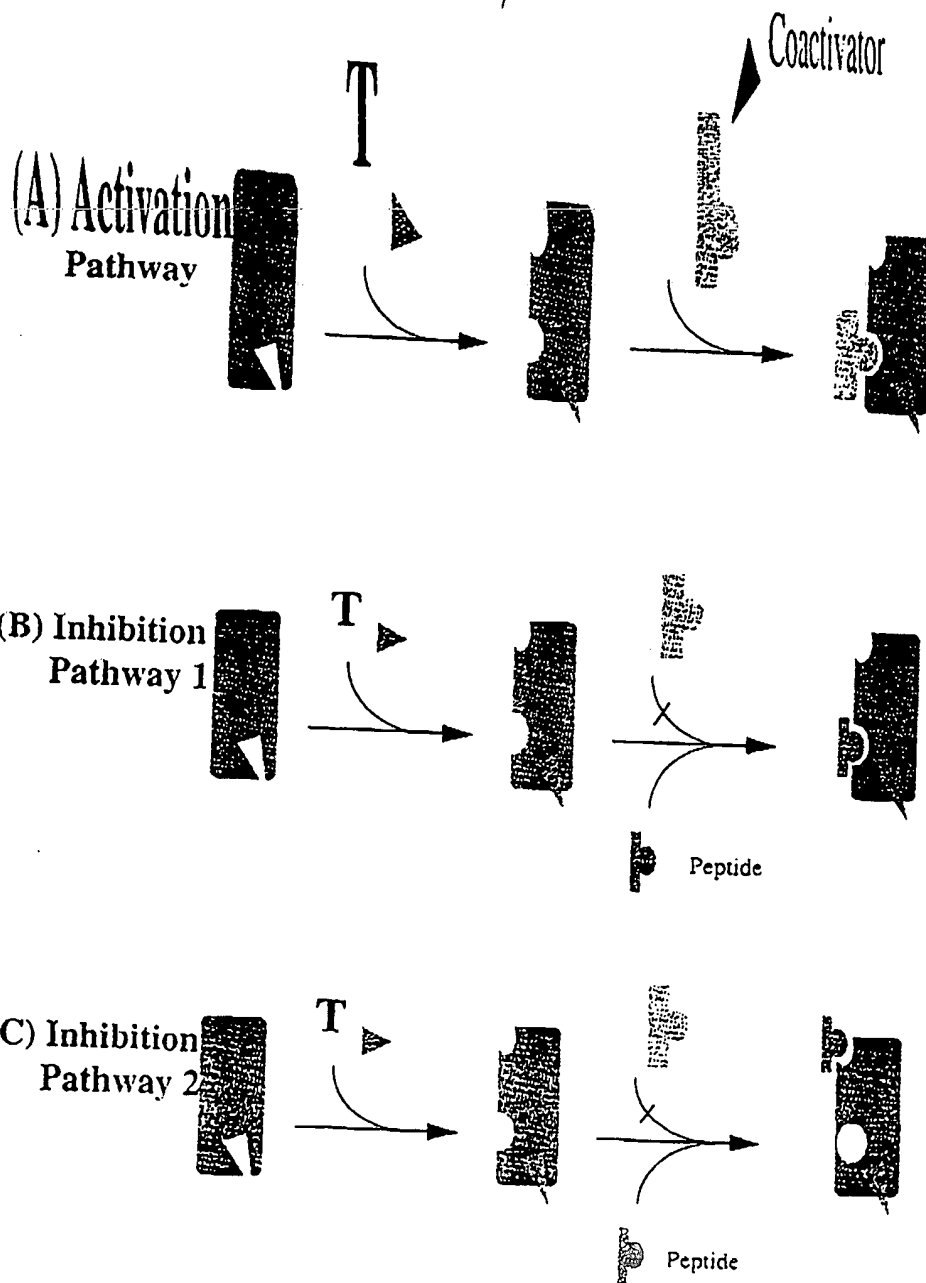


Figure 3. <sup>14</sup>LXXLL containing peptides are sensitive probes for ER A2 activation

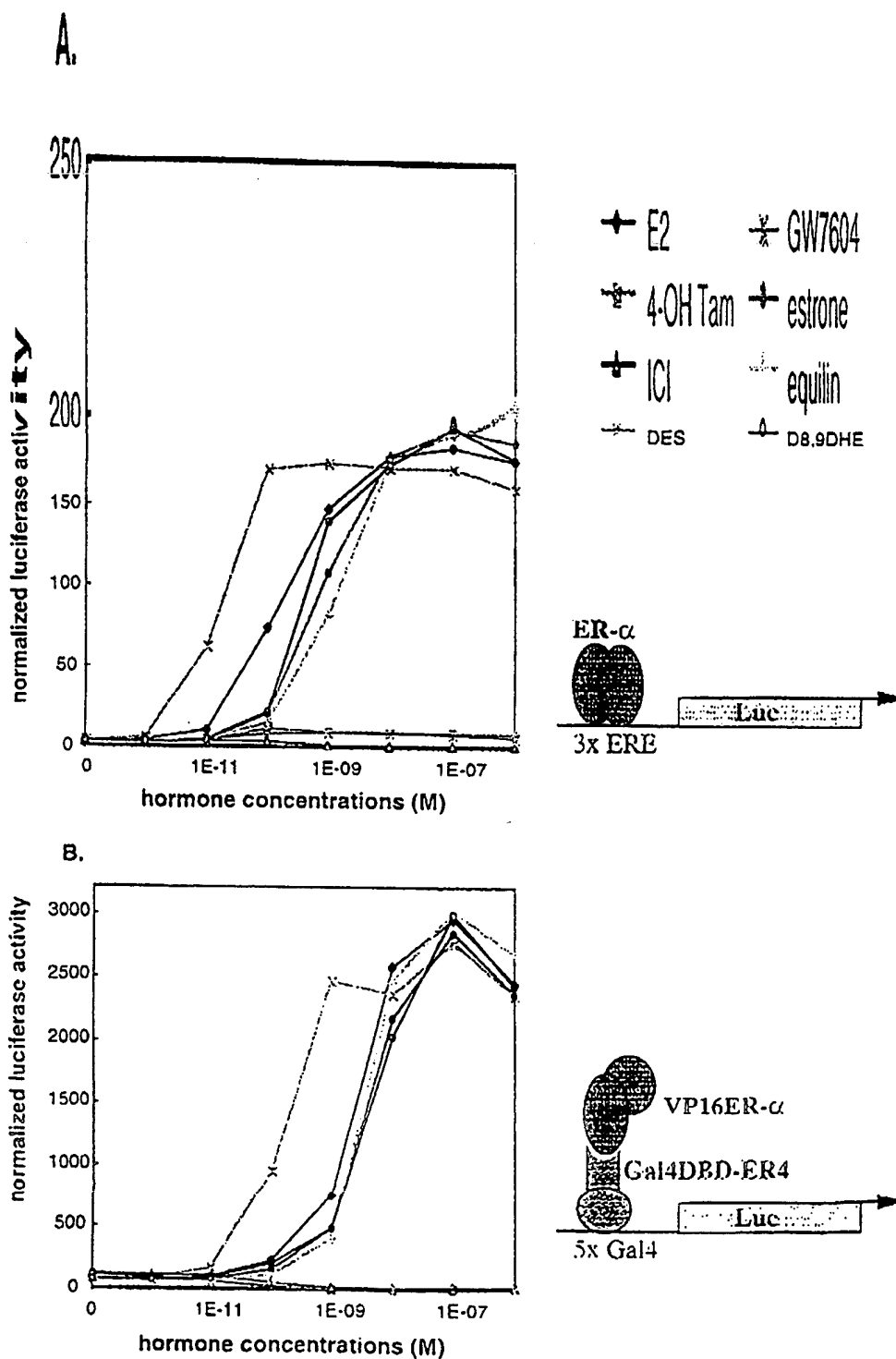
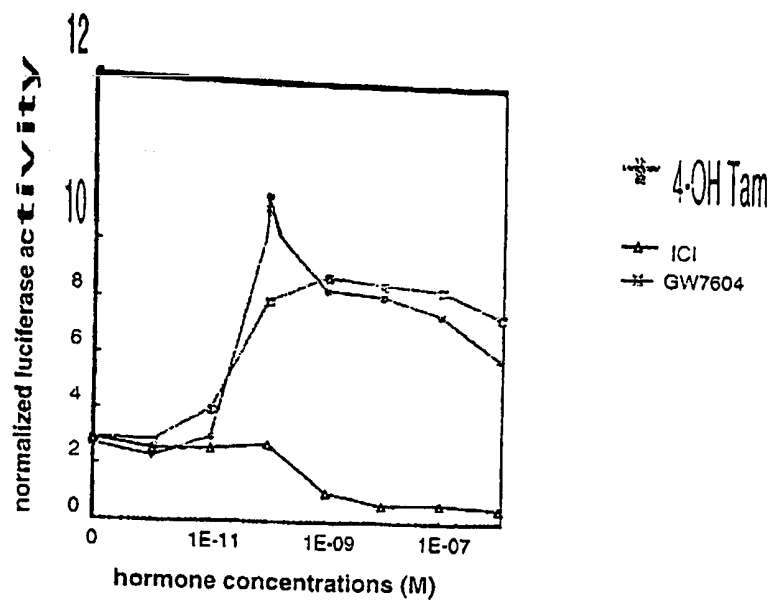




Fig. 14 (cont'd) Both partial agonists and pure antagonists  
disrupt ER-LXXLL motif interaction

C



D

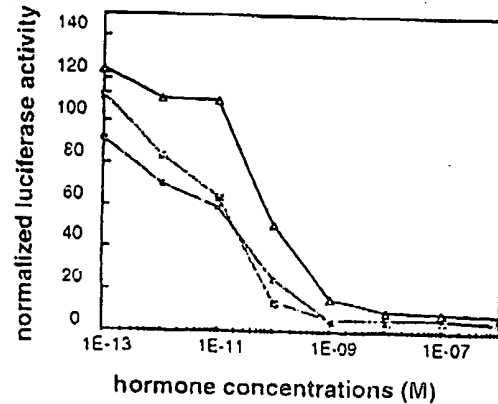
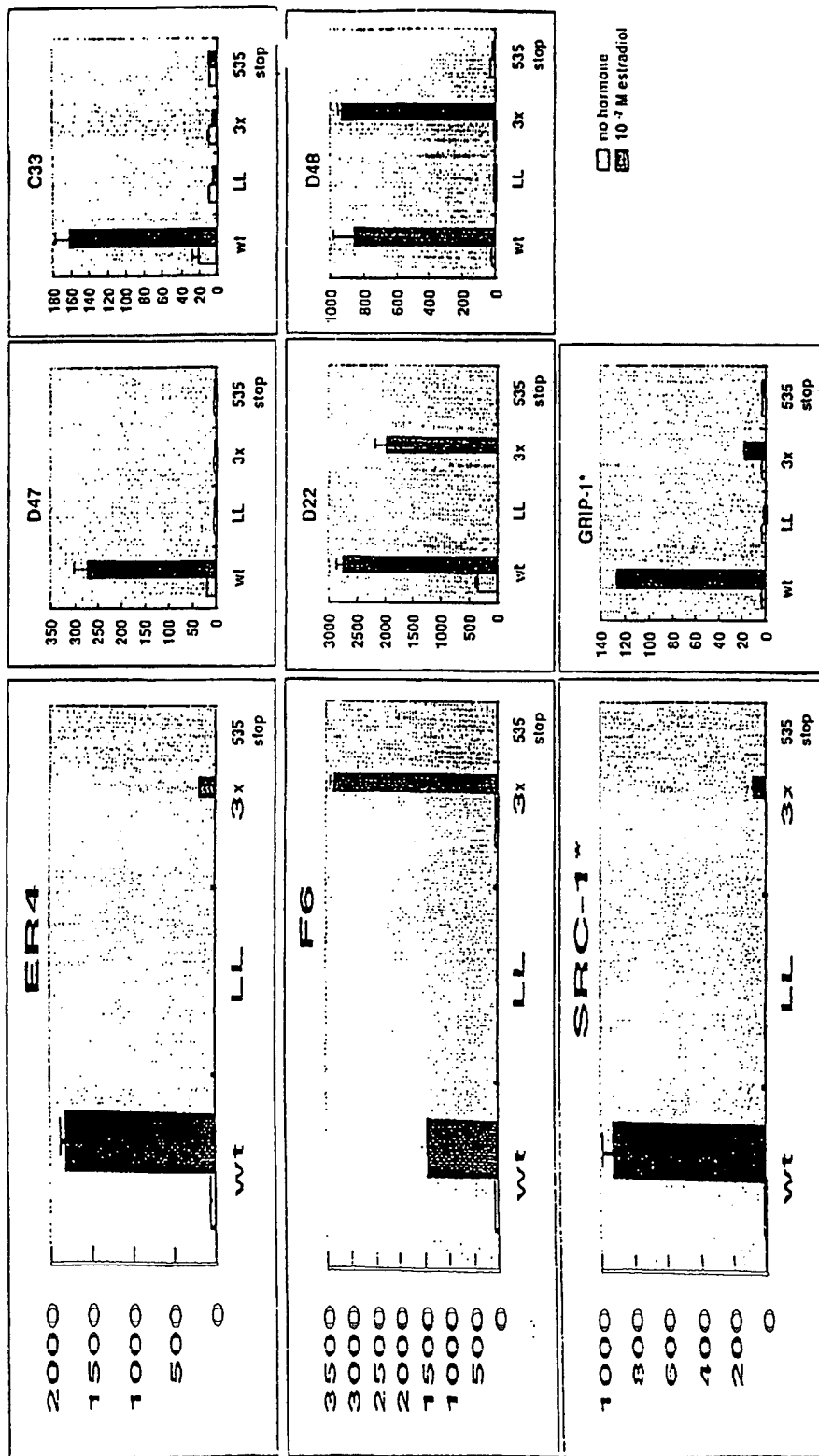


Fig. 15



**Figure 6. LXXLL containing peptides disrupt ER- $\alpha$  transcriptional activation function in mammalian cells**

(A)

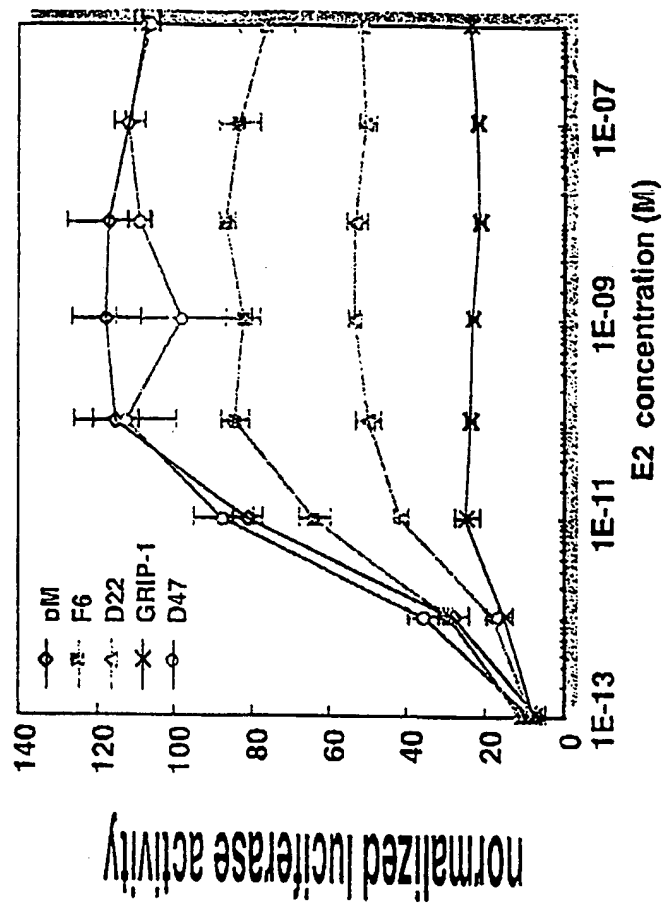
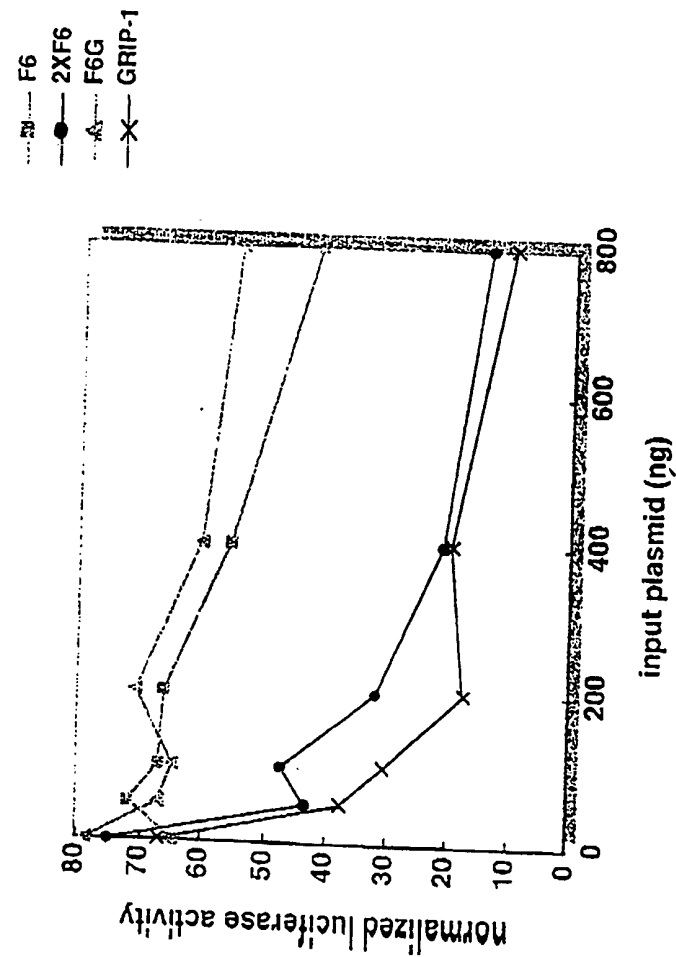


Fig. 16 (B)

Two copies of LXXLL motif function synergistically to compete with endogenous coactivators



(B)

**Figure 7. LXXLL containing peptides disrupt AF2 functions in HepG2 cells**

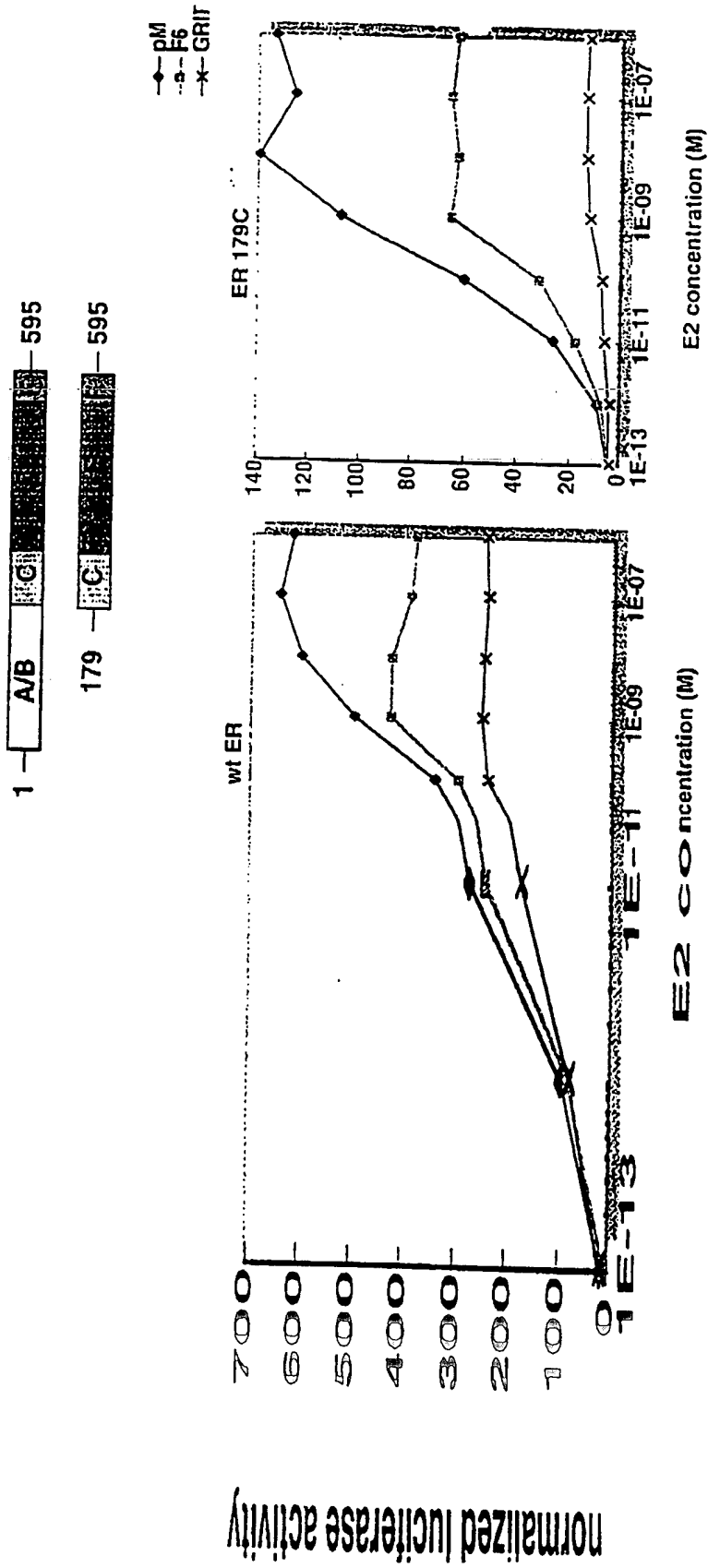
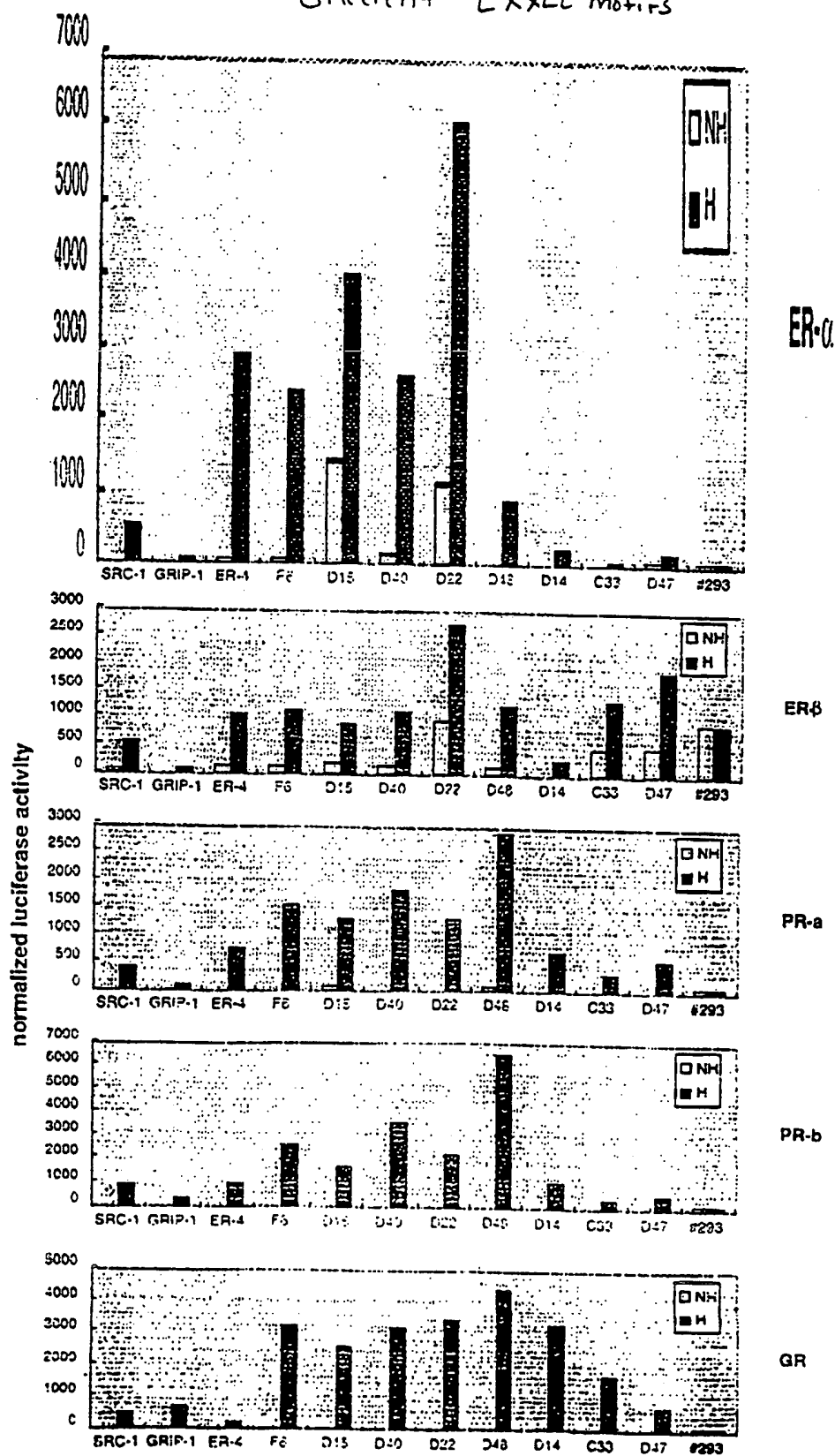


Fig 18. Nuclear receptors have different preferences for different LXXLL motifs



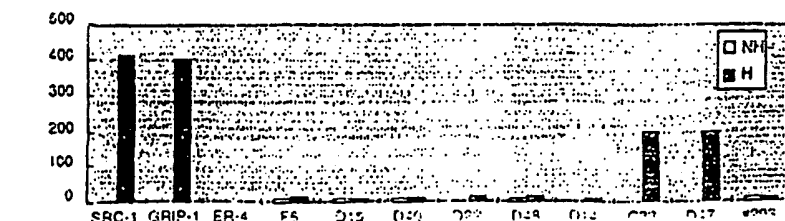
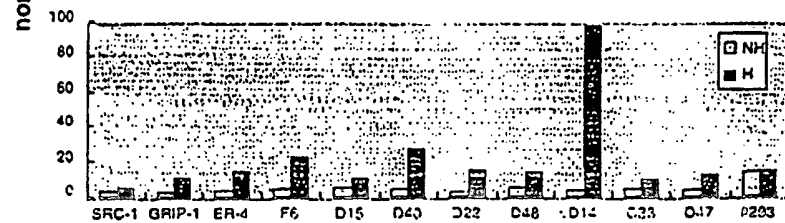
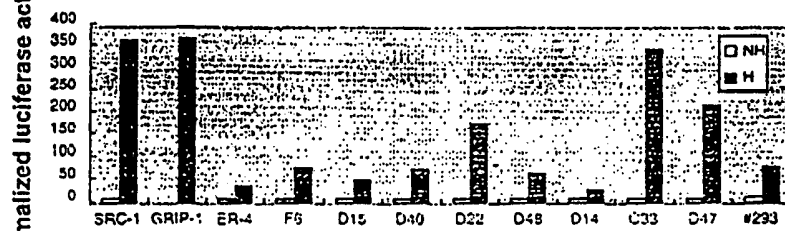
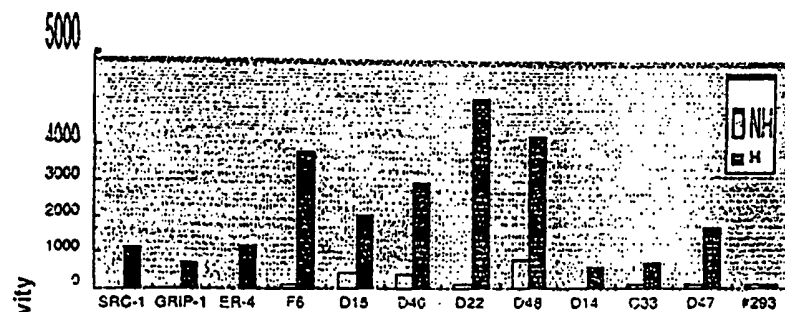
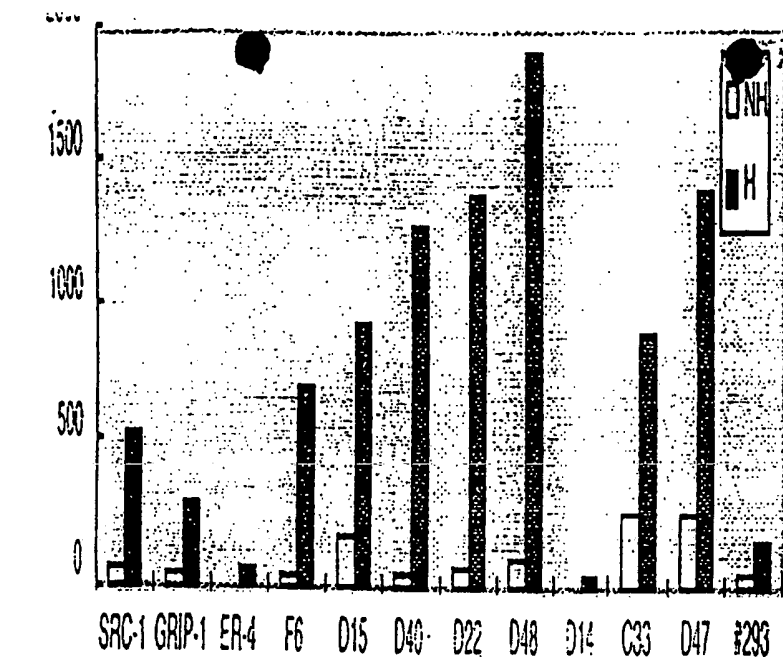


Fig. 20

	NH	E2	OT	ICI	Ralox	7804	Idox	Nafox
ntrol	1.341	1.621	3.076	0.845	2.031	1.414	2.294	2.053
1	293.5	5962	8.54	1.359	6.449	16.08	4.256	3.324
	752.3	4663	6821	507.3	5245	4805	6925	7397
3	32.93	34.67	4696	33.15	58.18	64.21	60.2	63.84
5	2.901	9.313	5837	7.16	5.059	13.2	21.75	8.434
l dist	0	6886.618	9627.63	381.3001	4501.922	4062.318	6179.562	6651.107
dist	6886.618	0	9787.266	7266.298	5983.961	5947.69	6372.767	6556.022
dist	9627.63	9787.266	0	9777.12	7617.299	7709.374	7437.648	7467.322
dist	381.3001	7266.298	9777.12	0	4737.759	4297.842	6417.774	6889.769
lox dist	4501.922	5983.961	7617.299	4737.759	0	440.2544	1680.089	2152.019
34 dist	4062.318	5947.69	7709.374	4297.842	440.2544	0	2120.054	2592.036
x dist	6179.562	6372.767	7437.648	6417.774	1680.089	2120.054	0	472.2028
fox dist	6651.107	6556.022	7467.322	6889.769	2152.019	2592.036	472.2028	0
l sim	1.00	0.58	0.42	0.98	0.73	0.75	0.63	0.60
sim	0.58	1.00	0.41	0.56	0.64	0.64	0.61	0.60
sim	0.42	0.41	1.00	0.41	0.54	0.53	0.55	0.55
sim	0.98	0.56	0.41	1.00	0.71	0.74	0.61	0.58
lox sim	0.73	0.64	0.54	0.71	1.00	0.97	0.90	0.87
34 sim	0.75	0.64	0.53	0.74	0.97	1.00	0.87	0.84
x sim	0.63	0.61	0.55	0.61	0.90	0.87	1.00	0.97
ox sim	0.60	0.60	0.55	0.58	0.87	0.84	0.97	1.00

$$\text{Max dist} = \text{SQRT}(7397 * 7397 * 5) = 16540.19$$

$$\text{Sim} = \frac{\text{maxdist} - \text{ligand dist}}{\text{maxdist}}$$